REMARKS

Favorable reconsideration of this application in view of the remarks to follow and allowance of the claims of the present application are respectfully requested.

Before addressing the issues raised in the Official Action, applicants have amended the claims in a manner as shown above. Support for the amendments is found at page 7, lines 21-25; page 8, lines 14-18; page 8, line 30 to page 9, line 9; page 9, line 22 to page 10, line 2; and page 10, lines 17 to 29.of the specification. Since no new matter has been introduced, entry thereof respectfully requested.

In the Official Action, regarding Claim 13, the Examiner requests applicant to recite the full name of MMDX then followed by the expression "(MMDX)".

In response, applicants have made the change accordingly as reflected by Claim 13, as presently amended.

Further, Claims 18-23 and 26-33 stand rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite.

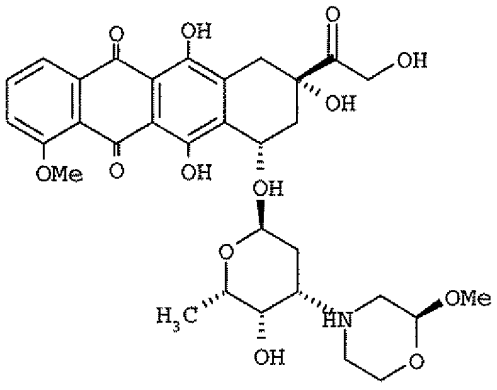
In response, applicants submit that the instant rejection has been obviated in view of the present amendments to the claims; therefore reconsideration and withdrawal of the same is respectfully requested.

Further, Claims 13-14 stand rejected under 35 U.S.C. §103(a) as allegedly unpatentable over U.S. Patent 5,304,687 to Bargiotti et al. ("Bargiotti et al.") in view of *Cancer Chemo. Pharm.*, 33, 10-16 (1993) to Kuhl et al. ("Kuhl et al.") and the English Abstract of *Gan. To Kagaku Ryoho.*, 8 Pt2, 2562-2567 (August 1988) to Nakamura et al., ("Nakamura et al.").

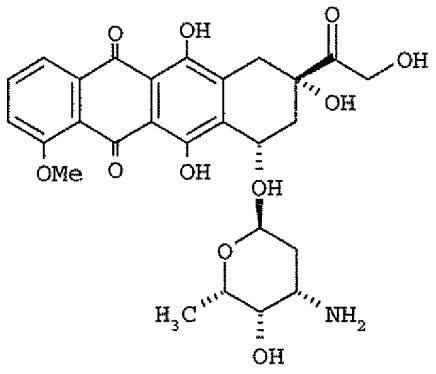
In response, applicants submit that Claims 13-14, as presently amended, are not obvious over the applied references for the reasons set forth below:

1. A person skilled in the art would not have reason or motivation to substitute Adriamycin by MMDX, and have a reasonable expectation of success that such modification would arrive at the presented claimed composition in treating liver cancer.

Applicants submit that MMDX is a new type of anti-tumor drug relative to Adriamycin and has a completely different mechanism of action than Adriamycin. Specifically, MMDX and Adriamycin are two different chemical entities. As a matter of fact, MMDX strongly differs from the most representative compound of the anthracycline class, Adriamycin, owing to the presence of a bulky methoxymorpholino group in place of the primary amino group in position 3' of the sugar moiety. To make immediately perceivable this difference, the chemical structures of the two compounds are shown below:



MMDX



ADR

Furthermore, it is well known that the pharmaceutical field is highly unpredictable. Such unpredictability substantially comes from differences of the chemical structures. Specifically, when the chemical structure is different, such structural difference will not only affect the physical and chemical properties of the compounds, but also it will profoundly affect the interaction between the compound and biological targets, so influencing the mechanism of action and, eventually, the biological activity of the compound. In this regard,

the structural difference of Adriamycin relative to MMDX has caused the two compounds to have completely different mechanisms of action. Specifically, it is well known that the activity of Adriamycin is due to its stimulation of DNA Topoisomerase II cleavage. On the contrary, MMDX has a completely different mechanism of action, as summarized below:

a. The bulky substituent at 3' position of MMDX totally inhibits the effects on the enzyme Topoisomerase II , thus the cytotoxic potencies of the MMDX are not related to Topoisomerase II inhibition. See the Abstract of Capranico et al., Molecular Pharmacology, Vol. 45, Issue 5, pp. 908-915, 1994 (referred to as "Exhibit 1").

b. MMDX interacts with DNA Topoisomerase I and stimulates DNA Topoisomerase I-induced cleavage at specific DNA sites. See the Abstract of van der Graaf et al., Cancer Chemoter Pharmacol (1995) 35: 345-348; the Abstract of Wassermann et al., Mol Pharmacol. 1990 July; 38(1): 38-45, Abst.; and the Abstract of Duran et al., Cancer Chemoter. Pharmacol., 1996, 38, p210-216 (referred to as "Exhibits 2-4" respectively).

c. MMDX exerts its cytotoxic effect through a mode of action different from that of "classical" anthracyclines and is not mediated through topoisomerase II inhibition. See Mariani et al., Invest New Drugs. 1994; 12(2): 93-7, Abst. (referred to as "Exhibit 5")

In view of the above remarks, applicants submit that the above publications demonstrate that MMDX is profoundly structurally and mechanistically different from Adriamycin and should not be grouped as one of various Adriamycin analogues but as a new type of anti-tumor drug. Therefore, a person skilled in the art would not have reason or

motivation to replace Adriamycin by MMDX, and have a reasonable expectation of success that such modification would be effective in treating liver cancer.

2. A person skilled in the art would not have reason or motivation to administer a composition into the hepatic artery to treat liver cancer where such composition comprises MMDX and a pharmaceutically acceptable agent which remains selectively in a liver tumor, after its injection into the hepatic artery.

Applicants submit that there is a technical prejudice in the art against the intra-arterial administration of anthracyclines (including Adriamycin) characterized by in vivo conversion into highly toxic metabolites. In this regard, *Pharmacokinetics and metabolism of anthracyclines*, Robert, Jacques; Gianni, Luca; Cancer Surveys (1993), 17 (Pharmacokinetics and Cancer Chemotherapy), 219-52 at page 229 (page 229 is to be provided in due course) provides:

> "Another approach, as expected from equation 1, would be that of using anthracyclines with total body clearances larger than those of doxorubicin and epirubicin. Idarubicin and iododoxorubicin would appear to be good candidates for this purpose. However, the faster clearance of the latter two anthracyclines is associated with an extensive metabolism to 13-dihydro metabolites, which are as active and as toxic as the parent drug.
>
> Since the liver is certainly involved in the metabolism of idarubicin and most likely plays a major part in the metabolism of iododoxorubicin, the application of equation 1 would be misleading and inappropriate, and the desired tumour selectively of the regional administration could be minimal. Furthermore, the hepatic intra-arterial administration of these anthracyclines could result in a systemic exposure to active and toxic metabolites even larger than that observed with intravenous administration, as already shown for doxorubicinol in patients with hepatoma (Lee et al., 1980). The desired tumour selectivity of the administration could then be **minimal**." (emphasis added)

In view of the above, it is clear that the art teaches away from the idea of hepatic intra-arterial administration of MMDX. As explained in the specification (see page 2), MMDX is converted in vivo into a metabolite having an activity and a toxicity 10-fold higher than that of

the parent compound. Therefore, a person skilled in the art would not even attempt to try to use MMDX for the local intrahepatic treatment of hepatic tumors, since MMDX would be converted there into a highly toxic metabolite, even in a larger amount than that observed with the systemic intravenous administration, and thus would cause significant toxicity to a human body.

3. None of the applied references recognize the unexpected result of the present invention.

Applicants submit that despite the fact that the art teaches away from hepatic intra-arterial administration of MMDX as demonstrated above, the present invention surprisingly recognizes that hepatic intra-arterial administration of MMDX to a patient suffering from a liver tumor reduces the amount of MMDX needed by other administration means (thus reducing the systemic exposure to toxic metabolite of MMDX) without decreasing the MMDX's antitumor activity at the hepatic tumor site.

In view of the above remarks, Applicants submit that the instant rejection has been obviated. Reconsideration and withdrawal of the instant rejection is respectfully requested.

Further, Claims 18-33 stand rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Bargiotti et al. in view of Kuhl et al., Nakamura et al., Gorbunova ("Gorbunova"), "Intrahepatic Arterial Infusion Chemotherapy for Primary and Metastatic Cancer of the Liver," (1990), and U.S. Patent No. 5,626,862 to Berm et al. ("Berm et al.").

In response, since Bargiotti et al., Kuhl et al. and Nakamura et al. have been cited in this rejection for the same reasons as in the 103 rejection to Claims 13-14, applicants submit that above remarks regarding the 103 rejection to Claims 13-14 are equally applied to the instant rejection and therefore are incorporated therein by reference. Specifically, applicants have shown that a person skilled in the art would not have reason or motivation to substitute

Adriamycin by MMDX, let alone administer a composition into the hepatic artery in treating liver cancer where such composition comprising MMDX and a pharmaceutically acceptable agent which remains selectively in a liver tumor after its injection into the hepatic artery.

Further, applicants submit that Gorbunova and Berm et al. not only fail to overcome the deficiencies of Bargiotti et al., Kuhl et al. and Nakamura et al. as discussed above, but also fail to teach or suggest the administration of MMDX by the time period and frequency as presently claimed.

Specifically, Gorbunova[1] discloses administration and dosage of Adriamycin and 5-fluorouracil for intrahepatic treatment of liver tumors. While Gorbunova teaches that intrahepatic administration creates super high concentration of an antitumor agent in the organ affected, it teaches that the time for intrahepatic administration is 72 hours for Adriamycin and 96 hours for 5-fluorouracil. See the second full paragraph at page 2 of Gorbunova. Gorbunova also teaches the specific side effects and disadvantages associated to this lengthy administration, for example, transient hepatic insufficiency, catheter thrombosis and patients forced to remain in a prone position in bed for the whole time, three or four days. See the last three full paragraphs at page 3 of Gorbunova. In contrast, the present invention has surprisingly and unexpectedly recognized that it is possible to administer MMDX in a much shorter time period (e.g., MMDX is administered as an infusion of from about 15 minutes to about 30 minutes every 4 weeks or as a 5-10 minute bolus every 8 weeks) with the very beneficial effect for the patients of avoiding long immobilization and thrombosis risk. Under the presently claimed method of intrahepatic treatment of tumors with MMDX, the patient can go home after treatment.

[1] A copy of an English translation is provided as Exhibit 6, and applicants' remarks are referred to this translated document.

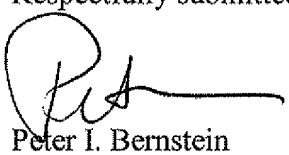
In view of the material and significant difference in terms of the administration time, e.g., 72 hours (4,320 minutes) vs. 15-30 minutes which is 144-fold less, as well as the disadvantages associated with the 72-hour administration and the benefits of the 15-30 minute administration, applicants submit that Gorbunova is not only far removed from the present invention, but also would discourage a person skilled in the art to apply intra-hepatic administration of MMDX in treating liver cancer.

Regarding Berm et al., applicants submit that it is further removed from the present invention because it does not disclose the intra-hepatic artery administration and it refers to a polymeric matrix which defines a different modality of drug release as opposed to the present invention.

In view of the above remarks, applicants submit that the applied references fail to present a *prima facie* case of obviousness. Therefore, Applicants submit that the instant rejection has been obviated. Reconsideration and withdrawal of the instant rejection is respectfully requested.

Thus, in view of the foregoing amendments and remarks, while Applicants fervently believe all claims are now allowable, to the extent any are so deemed by the Examiner, that these are identified and passed to issuance immediately. In the event that the Examiner has any queries concerning the instantly submitted Amendment, Applicants' attorney respectfully requests that he be accorded the courtesy of a telephone conference to discuss any matters in need of attention.

Respectfully submitted,



Peter I. Bernstein
Registration No. 43,497

Scully, Scott, Murphy & Presser, P.C.
400 Garden City Plaza, Suite 300
Garden City, New York 11530
516-742-4343
Enclosure: Exhibits 1-6

AZ/PIB:dk

EXHIBIT 1

0026-895X/94/050908-08$03.00/0

MOLECULAR PHARMACOLOGY, 45:908–915

Influence of Structural Modifications at the 3′ and 4′ Positions of Doxorubicin on the Drug Ability to Trap Topoisomerase II and to Overcome Multidrug Resistance

GIOVANNI CAPRANICO, ROSANNA SUPINO, MONICA BINASCHI, LAURA CAPOLONGO, MARIA GRANDI, ANTONINO SUARATO, and FRANCO ZUNINO

Division of Experimental Oncology B, Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milan, Italy (G.C., R.S., M.B., F.Z.), and Pharmacia Farmitalia-Carlo Erba Research Center, Experimental Oncology Laboratory, 20014 Nerviano, Italy (L.C., M.G., A.S.)

Received November 11, 1993; Accepted February 15, 1994

SUMMARY

To better define the role of the amino sugar in the pharmacological and biochemical properties of anthracyclines related to doxorubicin and daunorubicin, we have investigated the effects of various substituents at the 3′- and 4′-positions of the drug on cytotoxic activity and ability to stimulate DNA cleavage mediated by DNA topoisomerase II. The study shows that the nature of the substituent at the 3′-position but not the 4′-position is critical for drug ability to form cleavable complexes. The amino group at the 3′-position is not essential for cytotoxic and topoisomerase II-targeting activities, because it can be replaced by a hydroxyl group without reduction of activity. However, the presence of bulky substituents at this position (i.e., morpholinyl derivatives) totally inhibited the effects on the enzyme, thus supporting previous observations indicating that the cytotoxic potencies of these particular derivatives are not related to topoisomerase II inhibition. This conclusion is also supported by the observation that 3′-morpholinyl and 3′-methoxymorpholinyl derivatives are able to overcome atypical (i.e., topoisomerase II-mediated) multidrug resistance. Because a bulky substituent at the 4′-position did not reduce the ability to stimulate DNA cleavage, these results support a critical role of the 3′-position in the drug interaction with topoisomerase II in the ternary complex. An analysis of patterns of cross-resistance to the studied derivatives in resistant human tumor cell lines expressing different resistance mechanisms indicated that chemical modifications at the 3′-position of the sugar may have a relevant influence on the ability of the drugs to overcome specific mechanisms of resistance.

Like other intercalating agents with antitumor activity, anthracyclines exert their cytotoxic activity by interfering with DNA topoisomerase II function. Despite their apparent structural diversity, their molecular effects have been related to their ability to interfere with the breakage-rejoining action of topoisomerase II. Although the topoisomerase II inhibitors have a common intracellular target, the molecular basis of their variable therapeutic efficacy is still unknown. Similarly to other highly effective antitumor drugs, DOX stabilizes a transient DNA-topoisomerase II complex in which DNA strands are cut and covalently linked to the enzyme subunits (1–4). Investigations on the sequence specificity of DOX stimulation of *in vitro* DNA cleavage have led to a molecular model for drug action on topoisomerase II; drug molecules may be placed at the interface between the DNA cleavage site and the active site of the enzyme, thus forming DNA-drug-enzyme ternary complexes (5, 6).

The efficacy of DOX as an antitumor agent has stimulated many studies aimed at identifying critical substituents required for optimal activity. Previous studies on structure-activity relationships of anthracyclines have shown an important role for the structure and stereochemistry of the amino sugar (daunosamine) in the pharmacological and biochemical activity of anthracyclines related to DNR and DOX (7–10). The basic amino group at C-3′ has been implicated in determining the DNA binding affinity. However, the presence of a basic group at C-3′ is not a strict requirement for cytotoxic activity of anthracyclines (11). The role of substituents at the 3′-position remains unclear, because *N*-acyl derivatives exhibited low affinity for DNA and markedly reduced cytotoxic potency (7, 12), but substitution of the amino group for an hydroxyl group at C-3′ resulted in comparable cytotoxic activity (11).

The influence of selected chemical modifications at different

This work was partially supported by Consiglio Nazionale delle Ricerche and by Associazione Italiana per la Ricerca sul Cancro.

ABBREVIATIONS: DOX, doxorubicin; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; VM-26, 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside; SDS, sodium dodecyl sulfate; SV40, simian virus 40; MRP, multidrug resistance-related protein; DNR, daunorubicin; 4′-I-DOX, 4′-deoxy-4′-iododoxorubicin; ID_{50}, drug concentration inhibiting cell growth by 50%, compared with drug-free cultured cells; SCLC, small-cell lung cancer.

positions in the amino sugar has been examined with respect to the ability of the drug to trap DNA topoisomerase II (4, 13). In an attempt to better define the molecular pharmacology of anthracyclines, the present study was undertaken to examine the influence of various substituents at the 3′- and 4′-positions of DOX and/or DNR on the ability of the drugs to stimulate enzyme-mediated DNA cleavage. We provide evidence that (a) the 3′-position but not the 4′-position is critical for the ability of the drugs to interfere with topoisomerase II and (b) 3′-deamino-3′-hydroxy derivatives of DOX overcome *mdr1*-mediated but not atypical multidrug resistance.

Experimental Procedures

Materials. Anthracycline derivatives were synthetized at the Chemistry Department of Farmitalia-Carlo Erba (Milan, Italy). Drugs were dissolved in dimethylsulfoxide or deionized water at 0.1 mM, stored at −20° for a few weeks, and diluted in deionized water immediately before use. DNA topoisomerase II was purified from murine leukemia P388 cell nuclei by published procedures (14, 15) and was stored at −20° in 20 mM KH_2PO_4, pH 7.0, 50% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 1 mM β-mercaptoethanol. SV40 DNA, T4 polynucleotide kinase, agarose, and polyacrylamide were purchased from Bethesda Research Laboratories (Basel, Switzerland). [γ-^{32}P] ATP was purchased from Amersham (Milan, Italy). Calf intestinal phosphatase and restriction endonucleases were purchased from New England Biolabs (Taunus, Germany). Human *mdr1* and murine β-actin probes were as described previously (16).

Sequencing analysis of DNA cleavage sites. SV40 DNA fragments were uniquely 5′-end-labeled as described previously (5, 13). Briefly, SV40 DNA was cut with the indicated enzyme, dephosphorylated, and ^{32}P-labeled with T4 kinase. Then, DNA was subjected to a second enzyme digestion to generate uniquely 5′-end-labeled fragments, which were separated by agarose gel electrophoresis and purified by electroelution and ethanol precipitation. DNA cleavage reactions were performed in 20 μl of 10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, 15 μg/ml bovine serum albumin, with drugs, at 37° for 20 min. Topoisomerase II (106 units, about 200 ng of protein) was added in storage buffer (14). Reactions were stopped with SDS (1%) and proteinase K (0.1 mg/ml) and were incubated at 42° for 45 min. DNAs were then precipitated with ethanol, resuspended in 2.5 μl of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue, heated at 95° for 2 min, chilled on ice, and then loaded onto a 8% polyacrylamide denaturing gel. Gels were run at 70 W for 2 hr. Autoradiograms of dried gels were carried out using Amersham Hyperfilm.

Cell lines. POGB and POVD cell lines were obtained in our laboratory from lung tumor biopsies of two patients bearing SCLC. At the time of biopsies, patients had not been treated with chemotherapy or radiotherapy. After about 10 passages in RPMI 1640 medium supplemented with 10 nM hydrocortisone, 10 nM 17-β-estradiol, 30 nM sodium selenite, 5 mg/ml insulin, 100 mg/ml transferrin, and 5% FCS (Flow Laboratories), cells were adapted to grow in RPMI 1640 medium supplemented with 10% FCS (CM medium). POGB cells grew loosely attached to the flask and, when needed, they were detached through trypsinization or strong pipetting. POVD cells grew as floating clumps. After 10 passages in CM medium, cells were cultured in the presence of 1 ng/ml DOX; in subsequent passages, the DOX concentration was progressively increased up to 120 ng/ml for POGB cells and 100 ng/ml for POVD cells. Drug-selected cells were then tested for their sensitivity to DOX and were shown to have become resistant to this drug. The established resistant cell variants POGB/DX and POVD/DX were maintained always in the presence of 120 or 100 ng/ml DOX, respectively. One passage before each experiment, resistant cells were cultured in drug-free medium. The morphologies of resistant cells were similar to those of sensitive parental cells. The human leukemic cell line CEM and the vinblastine- and VM-26-resistant sublines CEM/VLB_{100} and CEM/VM1 (17–19) were kindly provided by Dr. W. T. Beck (St. Jude Children's Research Hospital, Memphis, TN). The cell lines were maintained at 37° in minimal essential medium (GIBCO) with Earle's salts, supplemented with 10% FCS and 1% vitamins (GIBCO), and were passaged twice weekly. Vinblastine (100 ng/ml) or VM-26 (66 ng/ml) was added at each passage to CEM/VLB_{100} and CEM/VM1 cells, respectively. Parent and resistant CEM cells grew as floating clumps.

Cytotoxicity test. SCLC cells (10^5/ml) were treated for 1 hr at 37° with drugs at different concentrations. Cells were then washed with phosphate-buffered saline and seeded in drug-free CM medium in 96-well tissue culture plates (10^4 POGB cells/well and 5×10^3 POVD cells/well). Ninety-six hours later cell survival was determined with the MTT assay, as described previously (20).

Immediately after seeding, CEM, CEM/VLB_{100}, and CEM/VM1 cells (2×10^4 cells/ml) were treated for 72 hr at 37° with drugs at different concentrations. Cell survival was then determined by cell counting. IC_{50} values were determined from the dose-effect curves.

Northern blot analysis. Total RNA was prepared by the LiCl-guanidine monothiocyanate method (21) from cells harvested in the logarithmic phase of growth. Total RNA (20 μg) was fractionated on a formaldehyde-containing 1% agarose gel and then transferred to a Hybond nylon membrane. The membrane was then irradiated with UV light, and prehybridization was performed for at least 4 hr at 42°, in 50% formamide, 5× standard saline citrate (i.e., 0.75 M NaCl, 75 mM Na citrate, pH 7.2), 0.2% SDS, 5× Denhardt's solution, 50 mM sodium phosphate, pH 7, 250 μg/ml salmon sperm DNA. DNA probes were ^{32}P-labeled with a random primer kit (specific activity, $2–5 \times 10^8$ cpm/μg of DNA). Hybridization was carried out for 20 hr at 42° in the same buffer containing 10% dextran sulfate.

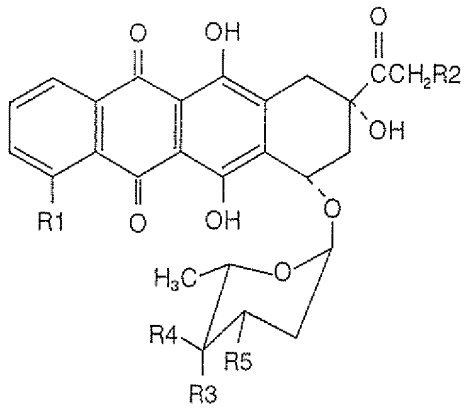
DNA binding studies. DNA binding parameters of anthracycline derivatives were determined by means of the fluorescence quencing method, as described previously (8–10), at the same ionic strength (0.1 M NaCl).

Results

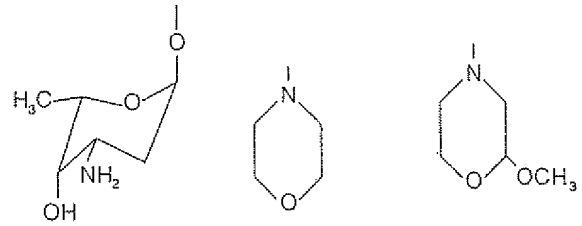
Stimulation of topoisomerase II DNA cleavage. The effect of various substituents at the 3′- and 4′-positions of the anthracycline molecule (Fig. 1) on the stimulation activity of topoisomerase II DNA cleavage was investigated with the experiments shown in Figs. 2 and 3. Drug stimulation of DNA cleavage was determined by incubating 5′-^{32}P-labeled SV40 DNA fragments with topoisomerase II and different concentrations of the analogs and analyzing DNA cleavage intensity patterns with polyacrylamide denaturing gels. Drug analogs were always compared with either DOX or 4-demethoxy-DNR, which have been shown to stimulate identical DNA cleavage intensity patterns (5).

3′-Morpholinyl and 3′-methoxymorpholinyl analogs of DOX did not stimulate enzyme-mediated DNA cleavage; however, they suppressed DNA cleavage at 1 and 10 μM (see 4880 site in Fig. 2). In contrast, 3′-deamino-3′-hydroxy-4′-morpholinyl-DOX stimulated DNA cleavage at the same sites and with similar relative intensities, compared with the parent drug (Fig. 2). Because the 3′-deamino-3′-hydroxy-4′-morpholinyl analog stimulated topoisomerase II DNA cleavage, the chemical nature of the morpholinyl group *per se* may not explain the inability of the 3′-morpholinyl derivatives to interfere with topoisomerase II function. Thus, the relative position of the substitution (3′ versus 4′) appeared to be the critical structural feature of the drug for retention of stimulation activity of DNA cleavage.

We then investigated the 3′-deamino-3′-hydroxy derivatives of DOX shown in Fig. 1. 3′-Deamino-3′-hydroxy-4′-amino-DOX and 3′-deamino-3′-hydroxy-4′-epi-DOX stimulated DNA cleavage to similar extents, compared with the parent



Compound	R1	R2	R3	R4	R5
Doxorubicin (DOX)	OCH_3	OH	OH	H	NH_2
Daunorubicin (DNR)	OCH_3	H	OH	H	NH_2
3'deamino-3'hydroxy -4'epiDOX	OCH_3	OH	H	OH	OH
4demethoxy-3'deamino-3'hydroxy-4'epiDOX	H	OH	H	OH	OH
3'deamino-3'hydroxy-4'aminoDOX	OCH_3	OH	NH_2	H	OH
3'morpholinylDOX	OCH_3	OH	OH	H	(b)
3'methoxy morpholinylDOX	OCH_3	OH	OH	H	(c)
3'deamino-3'hydroxy-4'morpholinylDOX	OCH_3	OH	(b)	H	OH
4'daunosaminylDNR	OCH_3	H	(a)	H	NH_2
4'I-DOX	OCH_3	OH	I	H	NH_2



(a) (b) (c)

Daunosaminyl Morpholinyl Methoxymorpholinyl

Fig. 1. Chemical structures of the studied anthracycline derivatives.

drug (Fig. 2). 4-Demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX was the most potent analog in stimulating DNA cleavage, because it was effective even at 0.1 μM. Because 4′-epimerization has been shown to have no effect on cleavage stimulation by anthracyclines (13), these observations indicated that the amino group at the 3′-position is not necessary for the drug effect on topoisomerase II. Indeed, lack of the amino group in the sugar increased the stimulation activity of anthracyclines for DNA cleavage (Fig. 2). 4′-Morpholinyl-DOX, 3′-deamino-3′-hydroxy-4′-epi-DOX, and 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX even at 10 μM stimulated DNA cleavage, whereas global suppression of DNA cleavage was observed at 10 μM for DOX, 3′-morpholinyl analogs, and 3′-deamino-3′-hydroxy-4′-amino-DOX (Fig. 2 and data not shown; see also Ref. 13). A lack of suppressive effect of the 3′-hydroxy derivatives may be rationalized in terms of reduced DNA binding affinity (Table 1), causing a different biphasic response.

To further evaluate the role of the 4′-position, we investigated 4′-*O*-daunosaminyl-DNR and 4′-I-DOX, which have an additional amino sugar and an iodine atom, respectively, at the 4′-position (Fig. 1). Both of these analogs stimulated cleavage of SV40 DNA in the presence of topoisomerase II (Fig. 3). 4′-I-DOX has been shown to stimulate protein-associated DNA breaks in living tumor cells (22).

DNA cleavage intensity patterns were identical among all of the studied analogs (Figs. 2 and 3). Weak cleavage sites were, however, more easily detected with the most potent derivatives (see sites from 4779 to 4814 in Fig. 2). The sequence specificity of DNA cleavage stimulation by the studied derivatives was thus the same as that reported for the parent drugs (5).

Cytotoxic potency of the anthracycline derivatives in human SCLC cell lines. All of the studied analogs were cytotoxic against human SCLC cells, although differences in potency could be noted. 3′-Deamino-3′-hydroxy-4′-epi-DOX was 3–4-fold less potent than DOX, and 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX was about as potent as the parent drug (Table 2). The increased potency of 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX, compared with 3′-deamino-3′-hydroxy-4′-epi-DOX, showed that removing the methoxy group at the 4-position in the planar ring system enhanced drug cytotoxic activity, in agreement with previous findings (23, 24). Because 4′-epimerization did not affect DOX cytotoxicity (8), removal of the 3′-amino group appears to be responsible for the reduced cell-killing activity. No precise correlation was found between the cytotoxic potency and DNA-cleaving activity, because these analogs were more active than DOX in stimulating topoisomerase II DNA cleavage (Fig. 2). Therefore, it may be possible that cellular pharmacokinetics of these analogs were somewhat altered by the chemical modification at the 3′-position, compared with DOX.

3′-Deamino-3′-hydroxy-4′-amino-DOX and 4′-*O*-daunosaminyl-DNR were somewhat less potent than DOX in SCLC cell lines (Table 2). However, they have been shown to be 2–3 times more potent than and as potent as the parent drug, respectively, in a sensitive CEM human leukemia cell line (25).

3′-Morpholinyl analogs were 3–6-fold more potent than DOX in the two sensitive SCLC cell lines, POGB and POVD (Table 2), in agreement with previous findings showing marked cytotoxic activity of 3′-morpholinyl-anthracyclines (26–28). In contrast, 4′-morpholinyl-DOX was similar to DOX, or slightly less potent than the parent drug (Table 2). Consistently, 4′-morpholinyl-DOX was 2.5-fold less potent, whereas 3′-morpholinyl analogs were about 4.5-fold more potent than DOX in an unrelated SCLC cell line, NCI-H187 (data not shown).

Cell-killing activity of drugs in human multidrug-resistant tumor cell lines with different mechanisms of resistance. The cytotoxic activities of the studied derivatives were also determined in the multidrug-resistant variants of the POGB and POVD SCLC lines (Table 2) and in two multidrug-resistant lines derived from a CEM human leukemia line (16–18) (Table 3). Both POVD/DX and POGB/DX lines were obtained from the corresponding sensitive lines by DOX selection (see Experimental Procedures for details).[1] It is likely that different mechanisms of drug resistance have been activated in these two DOX-selected SCLC sublines. Overexpression of the *mdr1* gene was found in POVD/DX cells, compared with the

[1] M. Binaschi, R. Supino, G. Capranico, and F. Zunino. Multidrug resistance in small cell lung cancer cell lines. Manuscript in preparation.

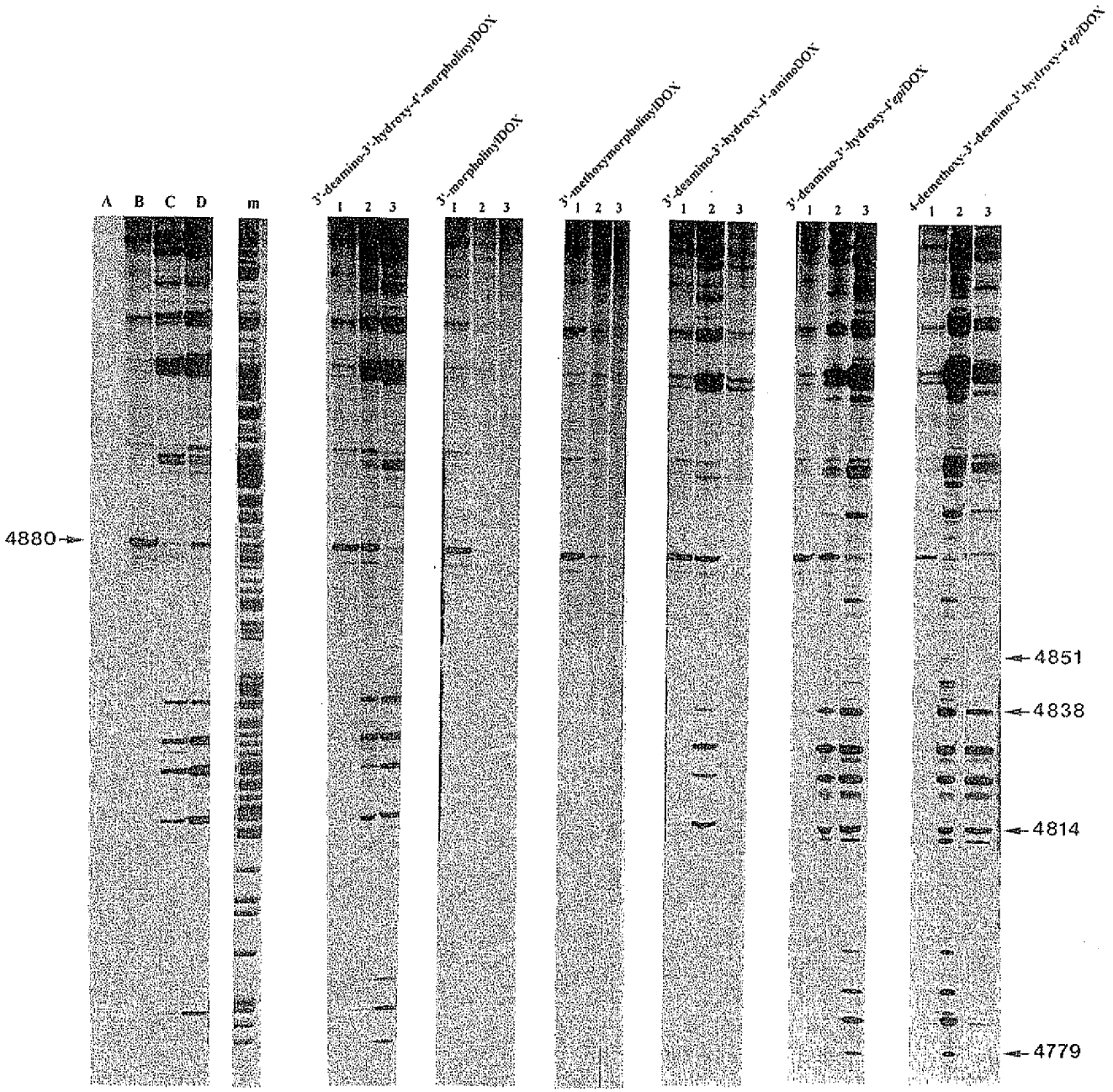


Fig. 2. Topoisomerase II DNA cleavage stimulated by the studied DOX derivatives in SV40 DNA. SV40 DNA ^{32}P-labeled at the *TaqI* site was incubated for 20 min at 37° with purified murine topoisomerase II and different concentrations of drugs. Cleavage reactions were stopped with 1% SDS and 0.1 mg/ml proteinase K, incubated at 42° for 45 min, precipitated with ethanol, and then analyzed on 8% polyacrylamide sequencing gels. *Lane A*, control DNA; *lane B*, topoisomerase II alone; *lane C*, with 1 μM DOX; *lane D*, with 1 μM 4-demethoxy-DNR; *lane m*, purine molecular weight markers; *lanes 1*, 2, and 3, 0.1, 1, and 10 μM indicated drug, respectively. *Arrows*, some cleavage sites; *numbers*, genomic position in SV40 DNA.

sensitive parental POVD cells (Fig. 4). In these experiments human colon cancer LoVo and LoVo/DX cells were used as a control system for P-glycoprotein overexpression (29). POVD/DX exhibited a pattern of cross-resistance typical of the multidrug-resistant phenotype, because it included vincristine, etoposide, and taxol but not cisplatin, melphalan, or 5-fluorouracil. In contrast, *mdr1* gene expression was not detected in POGB/DX cells, which instead exhibited amplification (about 50-fold) and overexpression of the MRP gene.[2] The phenotypes of multidrug-resistant CEM sublines have been shown to be distinct (17–19). CEM/VLB$_{100}$ cells had a classical multidrug-resistant phenotype and overexpressed the *mdr1* gene, whereas CEM/VM1 cells showed an atypical multidrug-resistant phenotype and did not overexpress the *mdr1* gene but instead had a mutated topoisomerase IIα gene coding for a drug-resistant protein (17–19).

The studied 3′-deamino-3′-hydroxy-4′-epi derivatives could overcome the drug resistance of POGB/DX, POVD/DX, and CEM/VLB cells but not that of CEM/VM1 cells (Tables 2 and 3). These observations suggested that an amino group in the sugar moiety of DOX might be critical for drug transport by P-glycoprotein. 3′-Morpholinyl derivatives were as active in all of the drug-resistant variant lines as in the corresponding

[2] M. Binaschi, R. Supino, R. A. Gambetta, G. Giaccone, E. Prosperi, G. Capranico, and F. Zunino. MRP gene overexpression and amplification in a human doxorubicin-resistant SCLC cell line.

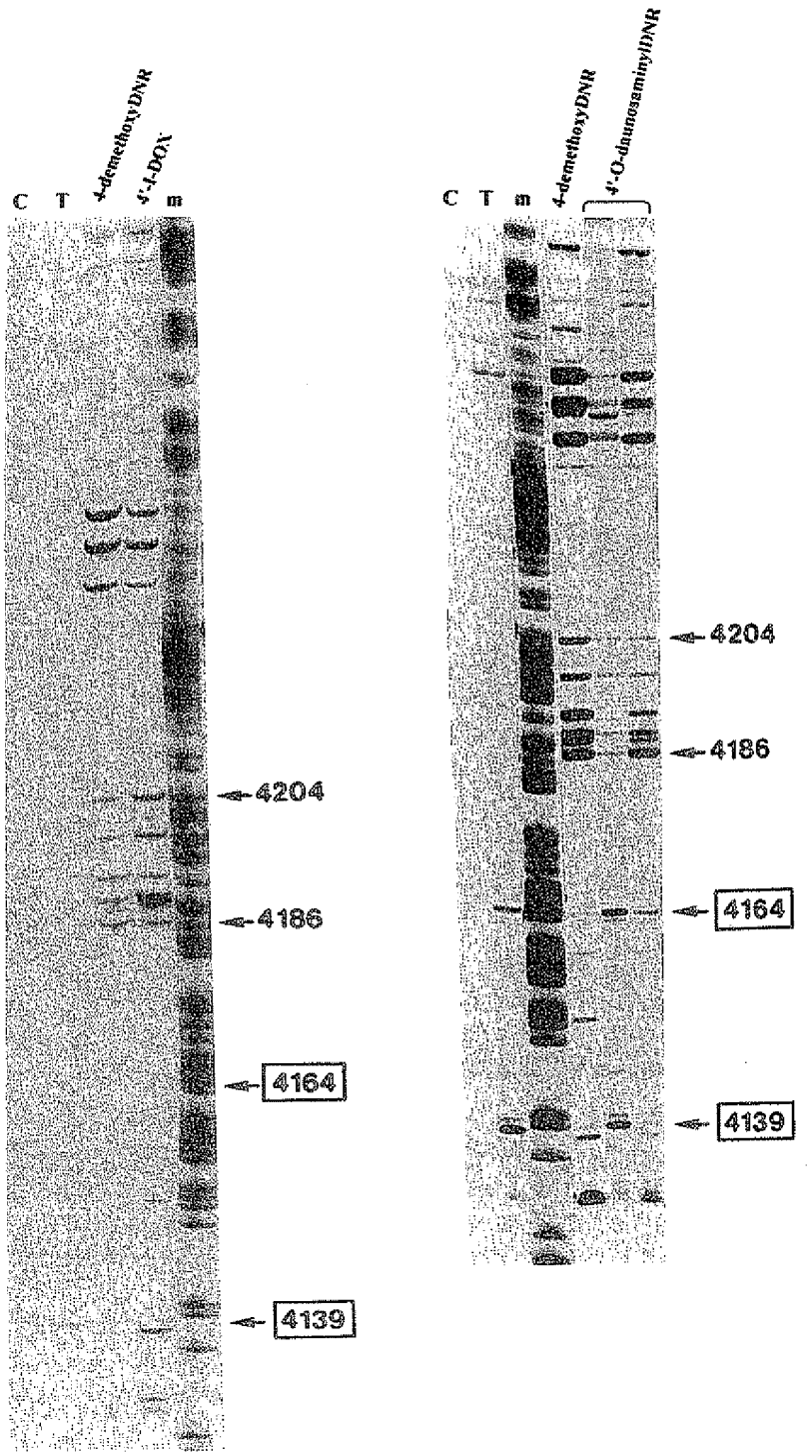


Fig. 3. Topoisomerase II DNA cleavage stimulated by the studied DOX derivatives in SV40 DNA. SV40 DNA was ^{32}P-labeled at a *Xho*II site. See legend to Fig. 2 for additional details. *Lane C*, control DNA; *lane T*, topoisomerase II alone; *lane m*, purine molecular markers. 4-Demethoxy-DNR and 4′-I-DOX were used at 1 μM. 4′-*O*-Daunosaminyl-DNR was used at 0.1 and 1 μM in the *left* and *right lanes*, respectively. *Arrows*, cleavage sites; *numbers*, genomic position in SV40 DNA. *Boxed numbers*, sites not stimulated by drugs.

parent cell lines. In contrast, CEM/VM1 but not CEM/VLB cells were fully cross-resistant to 3′-deamino-3′-hydroxy-4′-morpholinyl-DOX, supporting the idea that this derivative was directed against topoisomerase II also in living tumor cells.

The patterns of cross-resistance of these variant lines were not similar (Tables 2 and 3). Some observations can be made. (a) 3′-Morpholinyl derivatives were the only drugs showing no cross-resistance in the CEM/VM1 cell line, whereas all other drugs showed cross-resistance at similar or higher levels, compared with DOX (Table 3). (b) Only 4′-*O*-daunosaminyl-DNR and 3′-deamino-3′-hydroxy-4′-amino-DOX showed cross-resistance fully in CEM/VLB cells and at the highest level among the studied drugs in POVD/DX cells (Tables 2 and 3). Both of these analogs retained an amino group in the sugar moiety, and these two lines were drug resistant due to overexpression of the P-glycoprotein gene. (c) POGB/DX cells showed a specific pattern of cross-resistance; these cells were cross-resistant only to 3′-deamino-3′-hydroxy-4′-morpholinyl-DOX and 4′-*O*-daunosaminyl-DNR. This finding was consistent with a different mechanism of resistance developed by POGB/DX cells.

Discussion

Previous studies from this laboratory demonstrated the importance of the natural amino sugar (daunosamine) as a critical

TABLE 1

Binding parameters for the interaction of anthracycline derivatives with calf thymus DNA

K_{app} is the apparent binding constant; n is the apparent binding sites/nucleotide.

	K_{app} ($\times 10^6$)	n
	M^{-1}	
DNR	4.8	0.160
DOX	6.5	0.179
3′-Deamino-3′-hydroxy-4′epi-DOX	0.77	0.062
4-Demethoxy-3′-deamino-3′-hydroxy-4′epi-DOX	1.0	0.072
3′-Deamino-3′-hydroxy-4′amino-DOX	2.4	0.178
4′-I-DOX	6.4	0.100
4′-*O*-Daunosaminyl-DNR	2.7	0.283

determinant of anthracycline activity (8). In agreement with the view that DNA topoisomerase II is the primary target of drug action (4), we now provide evidence that the sugar moiety is also important for drug stimulation of topoisomerase II-mediated DNA cleavage. The 3′-*N*-substituted anthracyclines exhibited a reduced ability to stimulate topoisomerase II-mediated cleavage (30). The inability of 3′-morpholinyl and 3′-methoxymorpholinyl, but not 4′-morpholinyl, derivatives of DOX to stimulate enzyme-mediated DNA cleavage is consistent with these observations (see also Ref. 31) and suggests that a bulky substituent at the 3′-position is a steric hindrance for the formation of the ternary complex (drug-enzyme-DNA). Previous studies have emphasized the role of the amino group in the stabilization of the intercalation complex (32), and a free protonated amino group has been implicated in electrostatic interactions in the minor groove (33–35). Indeed, *N*-acetyl derivatives of DNR and DOX exhibited low DNA binding activity and markedly reduced cytotoxic and antitumor potencies. In contrast, because 3′-morpholinyl and 3′-methoxymorpholinyl derivatives are still very potent cytotoxic agents, it is evident that these compounds may exert cytotoxic activity by other mechanisms that are independent of topoisomerase II inhibition. 3′-Methoxymorpholinyl-DOX has a potential for covalent binding to DNA (36), and the formation of a drug-DNA adduct by active 3′-morpholinyl-DOX metabolites has been reported (27). 3′-Morpholinyl-DOX itself was found to retain DNA-binding ability and to stimulate topoisomerase I-induced DNA cleavage (31). Further investigations at a cellular level are required to better understand the mechanism of action of these derivatives.

The present study provides further evidence that an amino group at the 3′-position is not required to stimulate topoisomerase II-mediated DNA cleavage, because substitution of a hydroxyl group for the amino group resulted in compounds (i.e., 3′-deamino-3′-hydroxy-4′-epi-DOX and 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX) with activity comparable or superior to that of the corresponding 3′-aminoanthracyclines. This finding is consistent with previous observations that 3′-hydroxy derivatives retained cytotoxic and antitumor activities (11, 25). In contrast to 3′-morpholinyl-DOX, the compound with this substituent at the 4′-position has been found to have the same ability to trap topoisomerase II as exhibited by the parent compound, DOX. Similarly, different substitutions at the 4′-position (i.e., 4′-*O*-daunosaminyl-DNR and 4′-I-DOX) resulted in agents effective as topoisomerase II inhibitors. Overall, these results indicated that the presence of a bulky substituent at the 3′- but not the 4′-position prevents drug stimulation of topoisomerase II cleavage. As expected, the removal of the amino group in the sugar moiety caused an appreciable reduction of drug affinity for DNA. However, this reduction was accompanied by an appreciable increase in drug ability to trap DNA topoisomerase. A lack of precise correlation between the DNA binding affinity and the cellular and molecular effects of anthracyclines supports the view that the specific mode of DNA interaction is a more critical determinant for drug activity than is the strength of binding (4).

All of the tested 3′-hydroxy derivatives (Table 2) showed marked activity in the cytotoxicity assay. Using SCLC cell lines, the pattern of cross-resistance indicated that, with the exception of 3′-deamino-3′-hydroxy-4′-amino-DOX, all 3′-hydroxy derivatives overcame multidrug-resistance mediated by *mdr1* gene overexpression (i.e., POVD/DX cell line). It is possible that the presence of a free amino group is an important determinant for drug recognition by P-glycoprotein, because 4′-*O*-daunosaminyl-DNR also displays partial cross-resistance in this cell system. Alternatively, by removal of the sugar amino group the drug becomes more lipophilic, and this may favor cellular drug uptake by passive diffusion, thus counteracting the P-glycoprotein-dependent increased drug efflux in resistant cells. Similar results were obtained in the CEM/VLB cell line, with a typical multidrug resistant phenotype (Table 3).

The pattern of cross-resistance was somewhat different in the POGB/DX cell line. In this system, only compounds with a bulky substituent at the 4′-position showed cross-resistance. This subline exhibited a multidrug resistant phenotype with reduced intracellular drug accumulation, without *mdr1* expression.[2] It is possible that other transport systems (MRP gene

TABLE 2

Cytotoxic activities and cross-resistance of anthracycline derivatives in human SCLC cell lines

POVD/DX cells showed a classical multidrug-resistant phenotype and overexpressed the *mdr1* gene (Fig. 4); POGB/DX cells did not express the *mdr1* gene but had amplification and overexpression of the MRP gene.[2] IC_{50} values were determined from dose-response curves after 1-hr exposure to the drug. The shapes of the dose-response curves were similar for all tested compounds. In parentheses is the resistance index, calculated as the ratio of the IC_{50} values in DOX-resistant and parental cell lines.

Drug	IC_{50}			
	POGB	POGB/DX	POVD	POVD/DX
		$\mu g/ml$		
DOX	0.30	1.90 (6.3)	0.30	4.80 (16)
3′-Deamino-3′-Hydroxy-4′epi-DOX	1.22	2.00 (1.6)	1.00	2.10 (2.1)
4-Demethoxy-3′-deamino-3′-hydroxy-4′epi-DOX	0.22	0.25 (1.1)	0.41	0.25 (0.6)
3′-Deamino-3′-hydroxy-4′-amino-DOX	0.80	1.30 (1.6)	0.62	3.20 (5.2)
3′-Morpholinyl-DOX	0.12	0.14 (1.2)	0.05	0.17 (3.4)
3′-Methoxymorpholinyl-DOX	0.10	0.18 (1.8)	0.05	0.06 (1.2)
3′-Deamino-3′-hydroxy-4′-morpholinyl-DOX	0.38	1.55 (4.1)	0.70	1.20 (1.7)
4′-*O*-Daunosaminyl-DNR	0.88	3.95 (4.5)	0.76	3.65 (4.8)

TABLE 3

Cross-resistance to the studied anthracycline derivatives of two human multidrug-resistant CEM cell lines

CEM/VLB$_{100}$ cells showed a classical multidrug-resistant phenotype and overexpressed the *mdr1* gene, whereas CEM/VM1 cells showed an atypical multidrug-resistant phenotype and had a mutated topoisomerase IIα gene (16-18). Drug treatments were for 72 hr at 37°; drugs were then washed out, and cell survival was evaluated by cell counting. ID$_{50}$ values were 3.3, 72.3, and 28.7 ng/ml for 3′-deamino-3′-hydroxy-4′-amino-DOX and 6.1, 270, and 15.5 ng/ml for 4′-daunosaminyl-DNR in the parent CEM, CEM/VLB, and CEM/VM1 cell lines, respectively. ID$_{50}$ values for the other drugs were reported previously (24).

Drug	Resistance index relative to sensitive CEM line	
	CEM/VLB	CEM/VM1
DOX	31	5.7
4-Demethoxy-DNR	2.8	4.8
3′-Deamino-3′-hydroxy-4′epi-DOX	3.9	13.2
4-Demethoxy-3′-deamino-3′-hydroxy-4′epi-DOX	0.9	8.1
3′-Deamino-3′-hydroxy-4′-amino-DOX	22	8.7
3′-Morpholinyl-DOX	2.4	2.0
3′-Methoxymorpholinyl-DOX	1.0	1.1
3′-Deamino-3′-hydroxy-4′-morpholinyl-DOX	1.2	5.6
4′-O-Daunosaminyl-DNR	44	2.5
4′-I-DOX	1.2	2.9

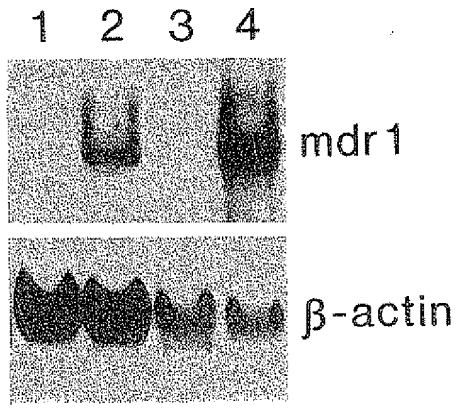


Fig. 4. Overexpression of the *mdr1* gene in DOX-resistant POVD/DX cells. Twenty micrograms of total RNA were fractionated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with the indicated human probes. *Lane 1*, POVD; *lane 2*, POVD/DX; *lane 3*, LoVo; *lane 4*, LoVo/DX.

product?) are involved, conferring a different cross-resistance pattern. Among the compounds examined, only 3′-morpholinyl and 3′-methoxymorpholinyl derivatives were found to be able to overcome resistance mediated by topoisomerase II gene mutations (i.e., in CEM/VM1 cells). This finding is in agreement with the hypothesis that these derivatives differ from conventional anthracyclines, with the natural amino sugar, in their mechanism of action (27). In CEM/VM1 cells all 3′-hydroxy derivatives were found to be cross-resistant, with a resistance index comparable or superior to that of DOX. Again, this observation is consistent with the conclusion that 3′-hydroxy derivatives exert cytotoxic activity through inhibition of topoisomerase II function. The inability of these compounds to overcome topoisomerase II-mediated resistance is expected on the basis of the identical sequence specificities of DNA cleavage stimulation, compared with DOX (5), suggesting a similar structural basis of drug interaction with topoisomerase II in the ternary complex.

Acknowledgments

We thank Dr. Don Phillips for stimulating and helpful discussion and L. Zanesi and L. Dal Bo for editorial and technical assistance, respectively.

References

1. Wang, J. C. DNA topoisomerases. *Annu. Rev. Biochem.* **54**:665–697 (1985).
2. Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **58**:351–375 (1989).
3. Pommier, Y., and K. W. Kohn. Topoisomerase II inhibition by antitumor intercalators and demethylepipodophyllotoxins, in *Developments in Cancer Chemotherapy* (R. I. Glazer, ed.). CRC Press, Boca Raton, FL, 175–195 (1989).
4. Zunino, F., and G. Capranico. DNA topoisomerase II as the primary target of anti-tumor anthracyclines. *Anticancer Drug Design* **5**:307–317 (1990).
5. Capranico, G., K. W. Kohn, and Y. Pommier. Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin. *Nucleic Acids Res.* **18**:6611–6619 (1990).
6. Capranico, G., and F. Zunino. DNA topoisomerase-trapping antitumour drugs. *Eur. J. Cancer* **28A**:2055–2060 (1992).
7. Arcamone, F. *Doxorubicin. Anticancer Antibiotics*. Academic Press, New York (1981).
8. DiMarco, A., A. M. Casazza, R. Gambetta, R. Supino, and F. Zunino. Relationship between activity and amino sugar stereochemistry of daunorubicin and Adriamycin derivatives. *Cancer Res.* **36**:1962–1966 (1976).
9. DiMarco, A., A. M. Casazza, T. Dasdia, A. Necco, G. Pratesi, P. Rivolta, A. Velcich, A. Zaccara, and F. Zunino. Changes of activity of daunorubicin, Adriamycin and stereoisomers following the introduction or removal of hydroxyl groups in the amino sugar moiety. *Chem. Biol. Interact.* **19**:291–302 (1977).
10. Zunino, F., A. DiMarco, A. Zaccara, and R. A. Gambetta. The interaction of daunorubicin and doxorubicin with DNA and chromatin. *Biochim. Biophys. Acta* **607**:206–214 (1980).
11. Priebe, W., N. T. Van, T. G. Burke, and R. Perez-Soler. Removal of the basic center from doxorubicin partially overcomes multidrug resistance and decreases cardiotoxicity. *Anti-Cancer Drugs* **4**:37–48 (1993).
12. Zunino, F., R. Gambetta, A. DiMarco, and A. Zaccara. Interaction of daunomycin and its derivatives with DNA. *Biochim. Biophys. Acta* **277**:489–498 (1972).
13. Capranico, G., F. Zunino, K. W. Kohn, and Y. Pommier. Sequence-selective topoisomerase II inhibition by anthracycline derivatives in SV40 DNA: relationship with DNA binding affinity and cytotoxicity. *Biochemistry* **29**:562–569 (1990).
14. De Isabella, P., G. Capranico, M. Binaschi, S. Tinelli, and F. Zunino. Evidence of DNA topoisomerase II-dependent mechanisms of multidrug resistance in P388 leukemia cells. *Mol. Pharmacol.* **37**:11–16 (1990).
15. Minford, J., Y. Pommier, J. Filipski, K. W. Kohn, D. Kerrigan, M. Mattern, S. Michaels, R. Schwartz, and L. A. Zwelling. Isolation of intercalator-dependent protein-linked DNA strand cleavage activity from cell nuclei and identification as topoisomerase II. *Biochemistry* **25**:9–16 (1986).
16. Supino, R., M. Binaschi, G. Capranico, R. A. Gambetta, E. Prosperi, E. Sala, and F. Zunino. A study of cross-resistance pattern and expression of molecular markers of multidrug resistance in a human small-cell lung-cancer cell line selected with doxorubicin. *Int. J. Cancer* **54**:309–314 (1993).
17. Beck, W. T., T. J. Mueller, and L. R. Tanzer. Altered surface membrane glycoproteins in *Vinca* alkaloid-resistant human leukemic lymphoblasts. *Cancer Res.* **39**:2070–2076 (1979).
18. Danks, M. K., J. C. Yalowich, and W. T. Beck. Atypical multiple drug resistance in a human leukemic cell line selected for resistance to teniposide (VM-26). *Cancer Res.* **47**:1297–1301 (1987).
19. Beck, W. T., M. C. Cirtain, M. K. Danks, R. L. Felsted, A. R. Safa, J. S. Wolverton, O. P. Suttle, and J. M. Trent. Pharmacological, molecular, and cytogenetic analysis of "atypical multidrug-resistant human leukemic cells." *Cancer Res.* **47**:5455–5460 (1987).
20. Supino, R., E. Prosperi, F. Formelli, M. Mariani, and G. Parmiani. Characterization of a doxorubicin-resistant murine melanoma line: studies on cross-resistance and its circumvention. *Br. J. Cancer* **54**:33–42 (1986).
21. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
22. Capranico, G., A. Riva, S. Tinelli, T. Dasdia, and F. Zunino. Markedly reduced levels of anthracycline-induced DNA strand breaks in resistant P388 leukemia cells and isolated nuclei. *Cancer Res.* **47**:3752–3756 (1987).
23. Supino, R., A. Necco, T. Dasdia, A. M. Casazza, and A. DiMarco. Relationship between effects on nucleic acid synthesis in cell cultures and cytotoxicity of 4-demethoxy derivatives of daunorubicin and Adriamycin. *Cancer Res.* **37**:4523–4526 (1977).
24. Capranico, G., C. Soranzo, and F. Zunino. Single-strand DNA breaks induced by chromophore-modified anthracyclines in P388 leukemia cells. *Cancer Res.* **46**:5499–5503 (1986).
25. Mariani, M., L. Capolongo, A. Suarato, A. Bargiotti, N. Mongelli, M. Grandi, and W. T. Beck. Growth-inhibitory properties of novel anthracyclines in human leukemic cell lines expressing either Pgp-MDR or at-MDR. *Invest. New Drugs*, in press.
26. Acton, E. M., G. L. Tong, C. W. Mosher, and R. L. Wolgemuth. Intensely potent morpholinyl anthracyclines. *J. Med. Chem.* **27**:638–645 (1984).

27. Lau, D. H. M., A. D. Lewis, and B. I. Sikic. Association of DNA cross-linking with potentiation of the morpholino derivative of doxorubicin by human liver microsomes. *J. Natl. Cancer. Inst.* **81**:1034–1038 (1989).
28. Ripamonti, M., G. Pezzoni, E. Pesenti, A. Pastori, M. Farao, A. Bargiotti, A. Suarato, F. Spreafico, and M. Grandi. *In vivo* anti-tumor activity of FCE 23762, a methoxymorpholinyl derivative of doxorubicin active on doxorubicin-resistant tumour cells. *Br. J. Cancer* **65**:703–707 (1992).
29. Ballinari, D., P. Radice, G. Sozzi, G. Pezzoni, P. Mondini, M. A. Pierotti, and F. C. Giuliani. MDR amplification and karyotypic analysis of anthracycline-resistant human tumor cell lines. *Cancer Commun.* **3**:69–74 (1988).
30. Bodley, A., L. F. Liu, M. Israel, R. Seshadri, Y. Koseki, F. C. Giuliani, S. Kirschenbaum, R. Silber, and M. Potmesil. DNA topoisomerase II-mediated interaction of doxorubicin and daunorubicin congeners with DNA. *Cancer Res.* **49**:5969–5978 (1989).
31. Wassermann, K., J. Markovits, C. Jaxel, G. Capranico, K. W. Kohn, and Y. Pommier. Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II. *Mol. Pharmacol.* **38**:38–45 (1990).
32. Zunino, F., R. Gambetta, A. DiMarco, A. Velcich, A. Zaccara, F. Quadrifoglio, and V. Crescenzi. The interaction of Adriamycin and its β anomer with DNA. *Biochim. Biophys. Acta* **476**:38–46 (1977).
33. Quigley, G. J., A. H. J. Wang, G. Ughetto, G. VanDerMarel, J. H. vanBoom, and A. Rich. Molecular structure of an anticancer drug-DNA complex: daunomycin plus d(CpGpTpApCpG). *Proc. Natl. Acad. Sci. USA* **77**:7204–7208 (1980).
34. Nakata, Y., and A. J. Hopfinger. Predicted mode of intercalation of doxorubicin with dinucleotide dimers. *Biochem. Biophys. Res. Commun.* **95**:583–588 (1980).
35. Wang, A. H.-J., G. Ughetto, G. J. Quigley, and A. Rich. Interactions between an anthracycline antibiotic and DNA: molecular structure of daunomycin complexed to d(CpGpTpApCpG) at 1.2-Å resolution. *Biochemistry* **26**:1152–1163 (1987).
36. Lau, D. H. M., A. D. Lewis, G. E. Duran, and B. I. Sikic. The cellular and biochemical pharmacology of the methoxy morpholino derivative of doxorubicin, FCE 23762. *Proc. Am. Assoc. Cancer Res.* **32**:332 (1992).

Send reprint requests to: G. Capranico, Istituto Nazionale Tumori, via Venezian 1, 20133 Milan, Italy.

EXHIBIT 2

Cancer Chemother Pharmacol (1995) 35: 345–348

SHORT COMMUNICATION

Winette T. A. van der Graaf · Nanno H. Mulder
Coby Meijer · Elisabeth G. E. de Vries

The role of methoxymorpholino anthracycline and cyanomorpholino anthracycline in a sensitive small-cell lung-cancer cell line and its multidrug-resistant but *P*-glycoprotein-negative and cisplatin-resistant counterparts

Received: 21 July 1993 / Accepted: 22 July 1994

stract The cytotoxic action of two morpholino anthracyclines, methoxymorpholino anthracycline (MRA-MT, FCE 23762) and cyanomorpholino anthracycline (MRA-CN), was compared with the cytotoxicity of doxorubicin (DOX), the topoisomerase II inhibitor etoposide (VP-16), the topoisomerase I inhibitor camptothecin, methotrexate, and cisplatin in GLC4, a human small-cell lung-cancer cell line, in GLC4-Adr, its P-glycoprotein (Pgp)-negative, multidrug-resistant (MDR; 100-fold DOX-resistant) subline with overexpression of the MDR-associated protein (MRP) and a lowered topoisomerase II activity, and in GLC4-CDDP, its cisplatin-resistant subline. GLC4-Adr was about 2-fold cross-resistant for the morpholino anthracyclines and GLC4-CDDP was, relative to GLC4, more resistant for the morpholino anthracyclines than for DOX. Overall, MRA-CN was about 2.5-fold more cytotoxic than MRA-MT. The cytotoxicity profile of the morpholino anthracyclines in these cell lines mimicked that of camptothecin.

y words Morpholino anthracyclines · MRP
isplatin resistance

Introduction

The development of multidrug resistance (MDR) is one of the major obstacles in successful chemotherapeutic treatment of cancer patients. One class of antitumor drugs with the widest spectrum of activity in human cancers comprises the anthracyclines, which, however, are involved in MDR [1]. For doxorubicin (DOX), one of the most frequently used drugs of this group of anthracyclines, several mechanisms contribute to its cytotoxic action. Cellular mechanisms of action include intercalation of DNA; the formation of DNA breaks, possibly due to the generation of free radicals; and the capacity to turn DOX-topoisomerase complexes into cellular poisons [2, 3]. Because of its toxicity, especially cardiac toxicity, and the appearance of drug resistance, new anthracycline analogs have been synthesized that are less toxic, more potent, and non-cross-resistant with DOX. One group of these analogs consists of compounds in which a morpholino ring incorporating the amino nitrogen of the daunosamine unit has been constructed. Acton et al. [4] synthesized a series of morpholino (e.g., MRA) and cyanomorpholino (e.g., MRA-CN) analogs of DOX. These morpholinyl-substituted anthracyclines have several properties in common that distinguish them from the parental anthracyclines. They are highly lipophilic, which facilitates rapid diffusion through the cell membrane [4, 5]. In contrast to DOX, MRA and MRA-CN are not cardiotoxic at effective antitumor doses [4, 6] and are not cross-resistant in DOX-resistant P-glycoprotein (Pgp)-positive and -negative cell lines [6–9]. Apart from its efficacy in Pgp-positive cell lines resistant to DOX, MRA-MT has also been proven effective in CEM/VM-1, a cell line with altered topoisomerase II, and in cell lines resistant to cisplatin and melphalan [10–13].

The working mechanisms of MRA and MRA-CN include preferential inhibition of ribosomal gene transcription [14] as well as topoisomerase I-mediated DNA cleavage [15]. This mechanism of cytotoxicity is probably different from that of DOX, which acts on topoisomerase II. Whereas MRA binds to DNA by intercalation and causes DNA strand breaks, MRA-CN produces DNA-DNA interstrand cross-links [15, 16]. It has been demonstrated that this interstrand DNA cross-link formation induced by MRA-CN, which takes place very rapidly, is preceded by the binding of drug to single-stranded DNA [17]. The marked difference in cytotoxicity and DNA-binding affinity observed between MRA and MRA-CN suggests a major role for the cyano substituent in the action of MRA-CN [18].

W. T. A. van der Graaf (✉) · N. H. Mulder · C. Meijer · E. G. E. de Vries
Department of Internal Medicine, Division of Medical Oncology, University Hospital, Oostersingel 59, 9713 EZ Groningen, The Netherlands

Table 1 ID_{50} values after 1 h incubation as determined in the MTA. Results are expressed as mean values (± SD) for 2–4 experiments performed in quadruplicate

	Cell lines		
	GLC4	GLC4-Adr	GLC4-CDDP
DOX (μM)	0.33 ±0.07	30.1 ±8.0	0.17 ±0.06
MRA-MT (μM)	0.0079±0.0017	0.020±0.005	0.030±0.009
MRA-CN (μM)	0.0045±0.0018	0.0052±0.0020	0.0088±0.0009

Table 2 ID_{50} values after continuous incubation as determined in the MTA. Results are expressed as mean values (± SD) for 2–7 experiments performed in quadruplicate

	Cell lines		
	GLC4	GLC4-Adr	GLC4-CDDP
DOX (nM)	32.5 ±2.1	3,732 ±336	42.3 ± 1.0
VP-16 (nM)	0.16±0.03	10.2 ± 2.4	0.11± 0.02
Camptothecin (nM)	6.1 ±2.5	7.6 ± 0.9	30.8 ±14.4
Methotrexate (μM)	0.05±0.03	0.14± 0.04	0.08± 0.02
Cisplatin (μM)	0.90±0.07	2.2 ± 0.2	11.9 ± 2.2
MRA-CN (nM)	0.59±0.16	1.4 ± 0.4	2.1 ± 1.4

In the present study the role of MRA-CN and MRA-MT in cell lines with well-defined, different patterns of resistance, namely, non-Pgp MDR and cisplatin resistance, was tested and compared with the cytotoxicity of these compounds in a sensitive cell line.

Materials and methods

GLC4 is a Pgp-negative human small-cell carcinoma cell line [19] and GLC4-Adr is the DOX-resistant subline of GLC4. It shows an atypical MDR phenotype with resistance to DOX, vincristine, VP-16, and m-AMSA without *mdr*-1 gene amplification or Pgp expression [20]. In GLC4-Adr, a membrane efflux pump different from Pgp and overexpression of a mainly cytoplasmic 110-kDa protein detectable with the monoclonal antibody LRP-56 as well as overexpression of the new putative membrane transporter gene MRP were demonstrated [21–23]. GLC4-CDDP is the cisplatin-resistant subline of GLC4 with a 13.2-fold resistance to cisplatin due to increased glutathione (GSH), unchanged glutathione S-transferase (GST), decreased DNA platination, and increased repair of platinum adducts [24, 25]. Topoisomerase II activity proved to be 100% in GLC4, 35% in GLC4-Adr, and 130% in GLC4-CDDP [26]. Topoisomerase I activity did not differ among these cell lines [20]. GSH levels are 2.5-fold higher in GLC4-CDDP and 2.1-fold higher in GLC4-Adr as compared with GLC4; GST activity is equal in GLC4 and GLC4-CDDP but is 1.7-fold higher in GLC4-Adr [25, 27]. The doubling times of these cell lines are as follows: GLC4, 16.5 h; GLC4-Adr, 21.8 h; and GLC4-CDDP, 28.0 h [25, 28]. All cell lines were cultured in RPMI 1640 medium and 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO_2.

A drug-sensitivity assay was performed with the microculture tetrazolium assay (MTA) as described previously [28]. To assure linearity the following numbers of cells per well (0.1 ml) were incubated: GLC4, 5,000; GLC4-Adr, 12,500; and GLC4-CDDP, 15,000. Cells were incubated with chemotherapeutic drugs either continuously for 4 days or for 1 h. When incubated for 1 h, the cells were washed. All assays were performed two to seven times in quadruplicate. The results are expressed as the mean (± SD) doses required to inhibit the growth of each cell line by 50% (ID_{50} values).

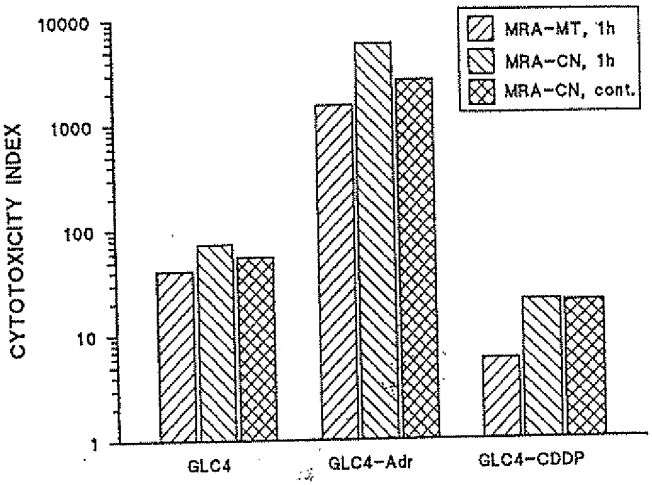


Fig. 1 Cytotoxicity index (ratio of the ID_{50} for DOX versus the ID_{50} for morpholino anthracyclines as determined in the MTA) obtained for each cell line

To measure the effect of buthionine sulfoximine (BSO) pretreatment on MRA-CN-induced cytotoxicity, GLC4, GLC4-Adr, and GLC4-CDDP cells were cultured for 48, 24, and 48 h, respectively, in the presence of 50 μM BSO without growth delay or loss of viability. Subsequently, MRA-CN-induced cytotoxicity (continuous incubation) was measured in the MTA (n = 3–4 experiments performed in quadruplicate).

Results

Tables 1 and 2 show the results of the 1-h and continuous incubations, respectively, in the MTA. Both morpholino anthracyclines are much more potent than DOX in these cell lines. The cytotoxicity of the morpholinyl derivates is remarkable in the highly DOX-resistant GLC4-Adr line. In contrast, GLC4-CDDP is relatively more sensitive to DOX but less sensitive to MRA-MT and MRA-CN than is GLC4. This comparison of the cytotoxicity sensitivity of the different cell lines for DOX, MRA-MT, and MRA-CN is expressed in Fig. 1. In this figure the cytotoxicity index is shown, which represents the ratio of the ID_{50} determined for DOX either after a 1-h incubation or after continuous incubation in a certain cell line versus the ID_{50} found for MRA-MT or MRA-CN. This demonstrates the potency of MRA-MT and MRA-CN with respect to DOX but also gives an impression about the mutual efficacy of the two morpholino anthracyclines and about the possibly different results of short versus continuous incubation. In all cell lines the cytotoxic action of the morpholino compounds surpassed the cytotoxicity of DOX. For both MRA-MT and MRA-CN the cytotoxicity indices were lowest in GLC4-CDDP and highest in GLC4-Adr. In the 1-h incubations, MRA-CN was 1.8-fold more active than MRA-MT in GLC4, 3.4-fold more active in GLC4-CDDP, and 3.8-fold more active in GLC4-Adr. In GLC4 and GLC4-Adr, 1-h incubations of MRA-CN seemed more effective than continuous incubations, whereas in GLC4-CDDP there was no difference.

Table 3 Cross-resistance factors[a] of GLC4-Adr and GLC4-CDDP versus GLC4 for DOX, MRA-MT, and MRA-CN

	Cell lines	
	GLC4-Adr	GLC4-CDDP
DOX	91	0.5
MRA-MT	2.5	3.8
MRA-CN	1.2	2.0

[a] At the ID_{50} as determined in the MTA (1 h incubation)

Table 4 Cross-resistance factors[a] of GLC4-Adr and GLC4-CDDP versus GLC4 for DOX, VP-16, camptothecin, methotrexate, cisplatin and MRA-CN

	Cell lines	
	GLC4-Adr	GLC4-CDDP
DOX	115	1.3
VP-16	64	0.7
Camptothecin	1.2	5.0
Methotrexate	2.8	1.6
Cisplatin	2.3	13.2
MRA-CN	2.4	3.6

[a] At the ID_{50} as determined in the MTA (continuous incubation)

The results of the MTA also led to cross-resistance factors, which are shown in Tables 3 and 4. The cross-resistance factors were calculated from the ratio of the ID_{50} determined for a certain chemotherapeutic drug in GLC-Adr and GLC4-CDDP, respectively, versus the ID_{50} in GLC4. The cross-resistance for DOX in GLC4-Adr was remarkably reduced for both morpholino compounds. This was also the case for camptothecin. GLC4-Adr showed some cross-resistance for methotrexate and cisplatin but was highly cross-resistant for VP-16. GLC4-CDDP was relatively insensitive to the morpholino anthracyclines as well as to camptothecin but was sensitive to DOX, VP-16, and methotrexate.

The effects of pretreatment with BSO on MRA-CN-luced cytotoxicity, expressed as dose-modifying factors at the ID_{50} as determined after continuous incubation in the MTA, were as follows: GLC4, 0.90 ± 0.06; GLC4-Adr, 1.04 ± 0.02; and GLC4-CDDP, 1.18 ± 0.26. Thus, BSO slightly increased MRA-CN-induced cytotoxicity in GLC4-CDDP cells.

Discussion

In our panel of cell lines, MRA-MT and MRA-CN are both very active chemotherapeutic drugs as compared with DOX. Both drugs are most active in GLC4-Adr, the cell line that is about 100-fold resistant to DOX. In this cell line, MRA-CN proved to be 5,790- and 2,590-fold more active than DOX after 1 h and continuous exposure, respectively. This is an interesting observation because of the remarkable properties of GLC4-Adr. Hence, in the MDR H69AR cell line, which also overexpresses MRP [29], Cole [30] reported a relative lack of potency for MRA-CN. However, in this cell line, no DOX-accumulation deficit exists [29]. From our observations the conclusion might be drawn that MRP, just as Pgp-mediated MDR, does not seem to be involved in the sensitivity to MRA-CN. Moreover, these morpholino anthracyclines circumvent altered topoisomerase II activity, as was demonstrated for their cytotoxic activity in the GLC4-Adr cell line. The relative lack of activity found for MRA-MT and, to a lesser degree, also for MRA-CN in GLC4-CDDP cells has not previously been described in a cisplatin-resistant cell line. Ripamonti et al. [13] reported an equivalent efficacy for MRA-MT in the wild-type and in the cisplatin-resistant murine leukemia cell line L1210. A 4-fold MRA-CN-resistant ES-2R cell line, however, also shows 7-fold cross-resistance to cisplatin [31]. The role of an enhanced amount of detoxifying activity, as has been reported for ES-2R as well as for GLC4-CDDP, might play a role in this cross-resistance between MRA-CN and cisplatin [31–33]. This was also partly suggested by the results of our experiments with BSO modulation on MRA-CN-induced cytotoxicity in GLC4-CDDP cells.

Concerning an elucidation of the working mechanisms of MRA-MT and MRA-CN in our cell lines, it is interesting that the cytotoxicity profiles of these drugs in the GLC4 cell lines mimic those of camptothecin and differ markedly from those of VP-16. This confirms earlier observations in which the cytostatic action of morpholino and cyano-morpholino doxorubicin was attributed to DNA topoisomerase I-induced cleavage and not to topoisomerase II-induced cleavage [15]. This finding has to be confirmed in other cell lines.

In our cell lines the activity of MRA-CN was about 2.5-fold that of MRA-MT. Previously, MRA-MT was reported to be 3- to 15-fold more potent than DOX in various cell lines, whereas MRA-CN was 100- to 1,000-fold more potent than DOX [9, 10]. We found an increase in the cytotoxicity of MRA-MT versus DOX that varied between 6- and 1,500-fold. The reason why our observations differ from the previous reports is not clear, as a modest difference in potency between MRA-MT and MRA-CN was observed in all our tested cell lines. The duration of incubation of MRA-CN made no uniform difference in its cytotoxic activity. Althoug the cross-link formation induced by MRA-CN takes place much faster than that caused by, e.g., cisplatin, this apparently has no effect on its final cytotoxic potential. Because of the generally promising cytostatic potency of the morpholino anthracyclines, the results of clinical studies, of which only a few have been reported to date, are awaited with great interest [34, 35].

In conclusion, MRA-MT and MRA-CN are highly potent chemotherapeutic drugs in a DOX-resistant cell line with overexpression of MRP and lowered topoisomerase II activity. Cross-resistance for the morpholino anthracyclines was found in a cisplatin-resistant cell line, suggesting a role for detoxifying systems such as GSH and GST. Topoisomerase I-mediated cytotoxicity is suggested because of the comparable cytotoxicity of the morpholino anthracyclines and camptothecin in the small-cell lung-cancer cell lines. MRA-CN is ca. 2.5-fold more active

than MRA-MT, whereas the duration of incubation does not play a uniform role in its cytotoxic potency.

Acknowledgement. This study was supported by grant GUKC 91-12 from the Dutch Cancer Society.

References

1. Bradley G, Juranka PF, Ling V (1988) Mechanism of multidrug resistance. Biochim Biophys Acta 948: 87
2. De Vries EGE, Zijlstra JG (1990) Morpholinyl anthracyclines: option for reversal of anthracycline resistance. Eur J Cancer 26: 659
3. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian topoisomerase II. Science 226: 466
4. Acton EM, Tong GL, Mosher CW, Wolgemuth RL (1984) Intensely potent morpholinyl anthracyclines. J Med Chem 27: 638
5. Streeter DG, Johl JS, Gordon GR, Peters JH (1986) Uptake and retention of morpholinyl anthracyclines by adriamycin-sensitive and -resistant P388 cells. Cancer Chemother Pharmacol 16: 247
6. Sikic BI, Ehsan MN, Harker WG, Friend NF, Brown BW, Newman RA, Hacker MP, Acton EM (1985) Dissociation of antitumor potency from anthracycline cardiotoxicity in a doxorubicin analog. Science 228: 1544
7. Coley HM, Twentyman PR, Workman P (1989) Identification of anthracyclines and related agents that retain preferential activity over adriamycin in multidrug-resistant cell lines, and further resistance modification by verapamil and cyclosporin A. Cancer Chemother Pharmacol 24: 284
8. Coley HM, Workman P, Twentyman PR (1991) Retention of activity by selected anthracyclines in a multidrug resistant human large cell lung carcinoma line without P-glycoprotein hyperexpression. Br J Cancer 63: 351
9. Streeter DG, Taylor DL, Acton EM, Peters JH (1985) Comparative cytotoxicities of various morpholinyl anthracyclines. Cancer Chemother Pharmacol 14: 160
10. Grandi M, Pezzoni G, Ballinari D, Capolongo L, Suarato A, Bargiotti A, Faiardi D, Spreafico F (1990) Novel anthracycline analogs. Cancer Treat Rev 17: 133
11. Grandi M, Mariani M, Ballinari D, Pezzoni G, Suarato A, Sreafico F, Chen M, Danks MK, Beck WT (1990) Lack of cross-resistance to certain anthracycline analogs in human leukemia multidrug resistant cells expressing either P-glycoprotein or altered DNA topoisomerase II. Proc Am Assoc Cancer Res 31: 2118
12. Lau DHM, Lewis AD, Durán GE, Sikic BI (1991) The cellular and biochemical pharmacology of the methoxymorpholino derivative of doxorubicin, FCE 23762. Proc Am Assoc Cancer Res 32: 1970
13. Ripamonti M, Pezzoni G, Pesenti E, Pastori A, Farao M, Bargiotti A, Suarato A, Spreafico F, Grandi M (1992) In vivo anti-tumour activity of FCE 23762, a methoxy-morpholinyl derivative of doxorubicin active on doxorubicin-resistant tumour cells. Br J Cancer 65: 703
14. Wassermann K, Newman RA, Davis FM, Mullins TD, Rose KM (1988) Selective inhibition of human ribosomal gene transcription by the morpholinyl anthracyclines cyanomorpholinyl- and morpholinyldoxorubicin. Cancer Res 48: 4101
15. Wassermann K, Markovits J, Jaxel C, Capranico G, Kohn KW, Pommier Y (1990) Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerase I and II. Mol Pharmacol 38: 38
16. Scudder SA, Brown JM, Sikic BI (1988) DNA cross-linking and cytotoxicity of the alkylating cyanomorpholino derivative of doxorubicin in multidrug-resistant cells. J Natl Cancer Inst 16: 1294
17. Lau DHM, Durán GE, Sikic BI (1992) Characterization of covalent DNA binding of morpholino and cyanomorpholino derivatives of doxorubicin. J Natl Cancer Inst 84: 1587
18. Chuang LF, Chuang RY, Acton EM, Israel M, Yu M (1987) Effect of morpholinyl-adriamycin analogs and adriamycin on the activities of DNA polymerase α and RNA polymerase II in chicken leukemia cells. J Pharmacol Exp Ther 242: 372
19. Zijlstra JG, Vries EGE de, Mulder NH (1987) Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res 47: 1780
20. De Jong S, Zijlstra JG, Vries EGE de, Mulder NH (1990) Reduced DNA topoisomerase II activity and drug-induced DNA cleavage activity in extracts from a human small cell lung carcinoma cell line. Cancer Res 50: 304
21. Scheper RJ, Broxterman HJ, Scheffer GL, Kaaijk P, Dalton WS, Heijningen THM van, Kalken C van, Slovak ML, Vries EGE de, Valk P van der, Meijer CJLM, Pinedo HM (1993) Overexpression of M_r 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res 53: 1475
22. Versantvoort CHM, Broxterman HJ, Pinedo HM, Vries EGE de, Feller N, Kuiper CM, Lankelma J (1992) Energy-dependent processes involved in reduced drug accumulation in multidrug-resistant human lung cancer cell lines without P-glycoprotein expression. Cancer Res 52: 17
23. Zaman GJR, Versantvoort CHM, Smit JJM, Eijdems EWHM, Haas M de, Smith AJ, Broxterman HJ, Mulder NH, Vries EGE de, Baas F, Borst P (1993) Analysis of the expression of MRP, the gene for a new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines. Cancer Res 53: 1747
24. Hospers GAP, Mulder NH, Jong B de, Ley L de, Uges DRA, Fichtinger-Schepman AMJ, Scheper RJ, Vries EGE de (1988) Characterization of a human small cell lung carcinoma cell line with acquired cisplatin resistance in vitro. Cancer Res 48: 403
25. Meijer C, Mulder NH, Hospers GAP, Uges DRA, Vries EGE de (1990) The role of glutathione in resistance to cisplatin in a human small cell lung cancer cell line. Br J Cancer 62: 72
26. De Jong S, Zijlstra JG, Mulder NH, Vries EGE de (1991) Lack of cross-resistance to fostriecin in a human small cell lung carcinoma cell line showing topoisomerase II related drug resistance. Cancer Chemother Pharmacol 28: 461
27. Meijer C, Mulder NH, Timmer-Bosscha H, Peters WHM, De Vries EGE (1991) Combined in-vitro modulation of adriamycin resistance. Int J Cancer 49: 582
28. Van der Graaf WTA, Vries EGE de, Uges DRA, Nanninga AG, Meijer C, Vellenga E, Mulder POM, Mulder NH (1991) In vitro and in vivo modulation of multidrug resistance with amiodarone. Int J Cancer 48: 616
29. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV, Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258: 1650
30. Cole SPC (1990) Patterns of cross-resistance in a multidrug-resistant small-cell lung carcinoma cell line. Cancer Chemother Pharmacol 26: 250
31. Lau DHM, Lewis AD, Ehsan MN, Sikic BI (1991) Multifactorial mechanisms associated with broad cross-resistance of ovarian carcinoma cells selected by cyanomorpholino doxorubicin. Cancer Res 51: 5181
32. Lau DHM, Ross KL, Sikic BI (1990) Paradoxal increase in DNA cross-linking in a human ovarian carcinoma cell line resistant to cyanomorpholino doxorubicin. Cancer Res 50: 4056
33. Lewis AD, Durán GE, Lau DH, Sikic BI (1992) Sensitization of drug resistant human ovarian cancer cells to cyanomorpholino doxorubicin (MRA-CN) by modulation of glutathione metabolism. Int J Radiat Oncol Biol Phys 22: 821
34. Majima H (1990) Phase I clinical study of MX2 (KRN-8602). Gan To Kagaku Ryoho 17: 359
35. Ogawa M, Tabata M, Horikoshi N, Inoue K, Mukaiyama T, Fukutani H, Hirano A, Muzunuma N, Itami S (1989) Phase I trial of 3′-deamino-3′-morpholino-13-deoxy-10-hydroxycarminomycin hydrochloride (KRN-8602). Proc Am Soc Clin Oncol 8: A238

EXHIBIT 3

Selected Abstracts

Returned: 1 citations and abstracts. **Click on down arrow or scroll to see abstracts.**

K Wassermann, J Markovits, C Jaxel, G Capranico, KW Kohn, and Y Pommier
Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II
Mol Pharmacol 38: 38-45.

Abstract 1 of 1

Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II

K Wassermann, J Markovits, C Jaxel, G Capranico, KW Kohn and Y Pommier

Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.

The effect of cyanomorpholinyldoxorubicin, morpholinyldoxorubicin, doxorubicin, and Actinomycin D were studied on purified mouse leukemia (L1210) DNA topoisomerases I and II. DNA unwinding and cross-linking were also studied. It was found that 1) morpholinyldoxorubicin, cyanomorpholinyldoxorubicin, and Actinomycin D (but not doxorubicin) stimulated DNA topoisomerase I-induced cleavage at specific DNA sites; 2) only doxorubicin and Actinomycin D stimulated DNA cleavage by DNA topoisomerase II; 3) at higher drug concentrations, DNA intercalators suppressed enzyme-mediated DNA cleavage induced by DNA topoisomerase I, as well as topoisomerase II; 4) only cyanomorpholinyldoxorubicin produced DNA-DNA cross-links; no DNA unwinding could be observed; and 5) DNA intercalation (unwinding) potency of morpholinyldoxorubicin was about 2-fold less than that of doxorubicin. The data indicate that some DNA intercalators are not only inhibitors of DNA topoisomerase II but act also on DNA topoisomerase I. The stabilization of cleavage intermediates by intercalators may have a common mechanism for DNA topoisomerase I and DNA topoisomerase II.

Volume 38, Issue 1, pp. 38-45, 07/01/1990

EXHIBIT 4

Cancer Chemother Pharmacol (1996) 38: 210–216

ORIGINAL ARTICLE

George E. Duran · Derick H.M. Lau
Alexander D. Lewis · Jörn-S. Kühl
Theodor K. Bämmler · Branimir I. Sikic

Differential single- versus double-strand DNA breakage produced by doxorubicin and its morpholinyl analogues

Received: 4 May 1995/Accepted 9 October 1995

Abstract The morpholinyl analogues of doxorubicin (DOX) have previously been reported to be non-cross-resistant in multidrug resistant (MDR) cells due to a lower affinity for P-glycoprotein relative to the parent compound. In order to further investigate the mechanisms of action of these morpholinyl anthracyclines, we examined their ability to cause DNA single- and double-strand breaks (SSB, DSB) and their interactions with topoisomerases. Alkaline elution curves were determined after 2-h drug treatment at 0.5, 2 and 5 μ*M*, while neutral elution was conducted at 5, 10 and 25 μ*M* in a human ovarian cell line, ES-2. A pulse-field gel electrophoresis assay was used to confirm the neutral elution data under the same conditions. Further, K-SDS precipitation and topoisomerase drug inhibition assays were used to determine the effects of DOX and the morpholinyl analogues on topoisomerase (Topo) I and II. Under deproteinated elution conditions (pH 12.1), DOX, morpholinyl DOX (MRA), methoxymorpholinyl DOX (MMDX) and morpholinyl oxaunomycin (MX2) were equipotent at causing SSB in the human ovarian carcinoma cell line, ES-2. However, neutral elution (pH 9.6) under deproteinated conditions revealed marked differences in the degree of DNA DSB. After 2-h drug exposures at 10 μ*M*, DSBs were 3300 rad equivalents for MX2, 1500 for DOX and 400 for both MRA and MMDX in the ES-2 cell line. Pulse-field data substantiated these differences in DSBs, with breaks easily detected after MX2 and DOX treatment, but not with MRA and MMDX. DOX and MX2 thus cause DNA strand breaks selectively through interaction with Topo II, but not Topo I. In contrast, MRA and MMDX cause DNA breaks through interactions with both topoisomerases with a predominant inhibition of Topo I.

Key words Anthracyclines · DNA strand breakage · Morpholinyl doxorubicin · MX2 · topoisomerases

Introduction

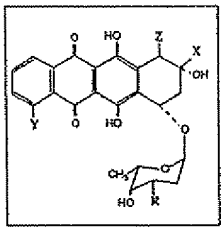
The antineoplastic agent, doxorubicin (DOX), is important clinically, but its use is limited due to cardiotoxicity, myelosuppression and multidrug resistance (MDR). In an attempt to overcome this drug resistance, Acton et al. [1] synthesized a series of DOX derivatives with morpholinyl groups at the 3′ position of the sugar moiety. These DOX analogues are non-cross-resistant in MDR variants in vitro, and have been shown to increase cellular accumulation of drug relative to the parent compound by tritiated and fluorescent assays [2–5]. These data suggest that the addition of the morpholinyl group alters the affinity of DOX for P-glycoprotein, an ATP-dependent efflux pump encoded by the *mdr*1 gene [6–10]. Also, these DOX analogues have been shown to be more lipophilic than DOX [1] and non-cardiotoxic at antitumor doses [2, 3]. Examples of these morpholinyl derivatives include morpholinyl DOX (MRA), methoxymorpholinyl DOX (MMDX), and morpholinyl oxaunomycin (MX2) (Fig. 1).

Previously, we have demonstrated that the MRA and MMDX compounds can be activated by microsomal metabolism and can crosslink DNA [11, 12]. This activation is associated with a potentiation of their cytotoxicity in vivo and in vitro [13, 14]. The closely related but extremely potent cyanomorpholinyl

Supported in part by NIH Grants CA 42417 and CA 52168, and American Cancer Society Grant CH-411 A

B.I. Sikic (✉)
Oncology Division, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305-5306, USA (Fax 415-725-1420)

Clinical Pharmacology Division, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305-5306, USA



	Name	Abbreviation	X	Y	Z	R
1.	Doxorubicin	DOX	-C(=O)-CH$_2$OH	-OCH$_3$	-H	-NH$_2$
2.	Morpholinyl doxorubicin	MRA	-C(=O)-CH$_2$OH	-OCH$_3$	-H	morpholinyl
3.	Morpholinyl oxaunomycin	MX2	-CH$_2$CH$_3$	-OH	-OH	morpholinyl
4.	Methoxymorpholinyl doxorubicin	MMDX	-C(=O)-CH$_2$OH	-OCH$_3$	-H	methoxymorpholinyl

Fig. 1 The chemical structures of DOX and the morpholinyl anthracyclines

derivative (MRA-CN) is capable of crosslinking DNA without microsomal activation [2]. We report here a further study of these native morpholinyl DOX compounds (i.e. no microsomal bioactivation). DNA elution assays were used in order to quantify the degree of single-strand breakage (SSB) and double-strand DNA breakage (DSB) induced after a 2-h treatment in a drug-sensitive human ovarian cell line relative to the parent compound, and the results were confirmed with a pulse-field gel electrophoresis assay. Further, data from K-SDS precipitation assays for protein–DNA complexes and topoisomerase activity inhibition experiments suggest that MRA and MMDX induce DNA strand breaks through interaction with topoisomerase I (Topo I), while the mechanism of action for MX2 appears to be more similar to DOX and related to the inhibition of topoisomerase II (Topo II).

Materials and methods

Drugs

MRA was generously provided by Dr. E. M. Acton (Drug Synthesis and Chemistry Branch, National Cancer Institute). MMDX was obtained from Farmitalia Carlo Erba Laboratories (Milano, Italy). MX2 was provided by the Kirin Company (Japan) and DOX was purchased as a commercial preparation from Adria Laboratories (Columbus, Ohio). Drug stock solutions were prepared in absolute ethanol at a concentration of 1.0 m*M* and stored at −20 °C.

Cell culture

The human ovarian carcinoma cell line, ES-2, was established in our laboratory and was grown as a monolayer culture in McCoy's 5A medium supplemented with 10% newborn calf serum, 0.3 mg/l glutamine, 100 U/ml penicillin/ml, and 100 mg/l streptomycin (all from GIBCO Laboratories, Grand Island, N.Y.). ES-2 cells were free of mycoplasma contamination as determined by the GEN-Probe hybridization assay (GEN-Probe, San Diego, Calif).

MTT cytotoxicity assay

The cytotoxicity of DOX and the morpholino anthracyclines was determined using a modified MTT 3-(4,5-dimethylthiazol(-2-yl,1-2,5-diphenyl-tetrazolium bromide assay [15]. ES-2 cells were plated in 96-well microtiter plates (Falcon, Becton Dickinson Co.; Lincoln Park, N.J.) in 200 µl medium. After 24 h, the cells were exposed to drugs at the appropriate dilutions and allowed to incubate for an additional 48 h (approximately two cell divisions). (MTT 20 µl, of MTT 5 mg/ml in phosphate-buffered saline) was added to each well and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 3 h, the medium was aspirated and 0.1 *N* HCl-isopropanol solution was added in order to solubilize the formazan crystals. Absorbances were read at 570 nm on a Molecular Devices U.V. Thermomax multiwell spectrophotometer (Molecular Devices, Menlo Park, Calif). Each drug was tested in quadruplicate and in at least three different experiments. Initial experiments indicated that 48 h was suitable for measuring cytotoxicity, as further drug incubation did not result in enhanced cytotoxicity.

Alkaline elution

The alkaline elution technique employed was modified from the method of Kohn [16]. Briefly, ES-2 cells were labeled for 36–48 h with 0.01 µCi/ml of [methyl-^{14}C]-thymidine and internal standard cells were labeled with 0.1 µCi/ml [methyl-^{3}H]-thymidine (both from Amersham Corporation, Arlington Heights, Il). The cells were chased with cold medium overnight and then exposed to drug for 2 h at 37 °C. In order to calculate radiation equivalents, one flask of ^{14}C-labeled cells was irradiated with 300 cGy using a mark 1, model 25 cesium-137 gamma-irradiating machine (J. L. Shepherd & Associated, Glendale, Calif). Internal control cells labeled with ^{3}H were irradiated with 400 cGy. Approximately 10 000 cpm ^{14}C-labeled cells and 20 000 cpm ^{3}H-labeled cells were loaded onto a smoke stack column with a 0.8 µm vinyl/acrylic copolymer filter (DM Metricel filter, 25 mm; Gelman Sciences, Ann Arbor, Mich). Cells were lysed by adding 2% SDS, 0.02 *M* EDTA at pH 10.0 in the presence and absence of 0.5 mg/ml proteinase K (Sigma Chemical Co., St. Louis, M.) for 30 min; the lysate on the filter was washed with 0.02 *M* EDTA, pH 10.0. The elution buffer consisted of 0.02 *M* EDTA and 0.1% SDS, adjusted to pH 12.1 with tetrapropylammonium hydroxide (Eastman Kodak Co., Rochester, N.Y.).

A Manostat cassette pump (New York, N.Y.) was employed to provide an elution rate of 2 ml/h and 4-ml elution fractions were collected using an LKB SuperRac fraction collector (LKB-Produkter, Bromma, Sweden). Ecolite scintillation cocktail (ICN Biomedicals, Irvine, Calif.) was added to each fraction, as well as to the *N* HCl-treated filter. Dual radioactivity was counted using an LS-8000 counter (Beckman Instruments, Palo Alto, Calif.). Log-fractions of ^{14}C retained against ^{3}H retained were plotted using an Excel program on a Macintosh computer.

Neutral elution

The alkaline elution procedure was followed with the following modifications. ^{14}C-labeled ES-2 cells were irradiated with 3000 cGy and ^{3}H-labeled cells with 5000 cGy. The elution buffer consisted of 0.02 *M* EDTA and 0.1% SDS, adjusted to pH 9.6 with tetrapropylammonium hydroxide for the detection of DSBS.

Asymmetric field inversion gel electrophoresis (AFIGE)

A modified AFIGE technique was followed for the quantification of DNA DSBs induced in ES-2 cells [17]. ES-2 cells were labeled with [methyl-^{14}C]-thymidine for 24 h followed by 2-h drug incubations. Cells were then cast in a 1% insert agarose (Seakem), and lysed in a 1% sodium lauryl sarkosine, 0.5 *M* EDTA and 1 mg/ml proteinase K solution at 50 °C overnight. The cells were treated with 100 µg/ml DNase-free ribonuclease A for 4 h at 37 °C. The 0.8% (w/v) agarose gel in 0.5 × Tris-borate/boric acid (TBE) buffer was run at 900s forward pulse time at 1.25 V/cm and 75 s backward pulse time at − 2.5 V/cm for a total electrophoresis time of 33 h. The agarose plugs were then removed from the gel, treated with 10 *N* HCl and melted on a hotplate. Ecolite scintillation cocktail was added to each and read in a Beckman LS-8000 counter. Quantification of DNA DSBs was determined by calculating the percentage of DNA released from the agarose plugs using an Excel program on a Macintosh computer.

K-SDS precipitation assay

The topoisomerase assay of Rowe et al. [18] was used with some modification. Briefly, ES-2 cells were labeled with 1 µCi/ml of [methyl-^{3}H]-thymidine for 24 h. Cells were then washed once in PBS and exposed to the appropriate drug dilution (10 µ*M* for each cytotoxin tested) for 1 h at 37 °C in an atmosphere containing 5% CO_2. The medium was subsequently aspirated and cells lysed with a 1.25% SDS, 5 m*M* EDTA (pH 8.0) supplemented with 0.4 mg/ml salmon sperm DNA. After shearing the DNA, cell lysates were transferred to 1.5-ml Eppendorf tubes containing 200 µl 325 m*M* KCl. Each tube was maintained at 65 °C for 5 min and then cooled on ice for an additional 10 min. Tubes were then spun at 10000 *g* for 2 min at room temperature in an Eppendorf centrifuge. The supernatants were aspirated and pellets washed twice with 10 m*M* Tris-HCl (pH 8.0), 100 m*M* KCl, 1 m*M* EDTA, supplemented with 0.1 mg/ml salmon sperm DNA. The suspension was cooled on ice and spun at 10000 *g*. The pellet was resuspended in 500 µl H_2O and maintained at 65 °C until the pellet was dissolved. After the suspension was transferred to a scintillation vial, 10 ml Ecolite cocktail was added to each and was counted in a Beckman LS-8000 counter. The percentage of specifically precipitated DNA was calculated using an Excel program on a Macintosh computer as follows:

$$\% \text{ precipitated} = \frac{\text{Counts}_{\text{tested}} - \text{Counts}_{\text{control}}}{\text{Counts}_{\text{plated}} - \text{Counts}_{\text{control}}} \times 100$$

Topoisomerase I/II drug screening

The induction of cleavage complexes by Topo I and II was studied using drug screening assay kits purchased from TopoGEN (Columbus, Ohio). Briefly, purified human DNA Topo I (10 units) was incubated with 0.25 µg of supercoiled *pHOT* plasmid (form I DNA) with a specific cleavage site for Topo I in a buffer containing 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA and 100 m*M* NaCl. Drugs were added to the mixture to final concentrations of 0.1, 0.5, 0.75 and 1 µ*M* and incubated at 37 °C for 30 min in a Perkin Elmer 9600 DNA thermal cycler (Norwalk, Ct.). Camptothecin (CPT) was used as a positive control drug for the inhibition of Topo I activity. The reaction mixture (20 µl) was stopped by the addition of 10% SDS and proteinase K (50 µ g/ml) followed by extraction with chloroform and isoamyl alcohol (24: 1 v/v).

For the Topo II assays, 4 units of human Topo II was incubated with a supercoiled DNA substrate (*pRYG* DNA) containing a single Topo II cleavage site in the presence and absence of drug for 30 min at 37 °C. VM-26, a known Topo II inhibitor, was used as a positive control. The buffer for the Topo II assays consisted of 30 m*M* Tris-HCl (pH 7.6), 3 m*M* ATP, 15 m*M* mercaptoethanol, 8 m*M* $MgCl_2$ and 60 m*M* NaCl. The assay was extracted as described above and the samples were then analyzed by agarose (1%) gel electrophoresis at 1.5 V/cm in 4 × Tris-acetate/EDTA (TAE) buffer. DNA was visualized by ethidium bromide staining.

Results

The in vitro cytotoxicity data for DOX and its morpholinyl derivatives in the ES-2 cell line are summarized in Table 1. MMDX was the most potent, followed by MRA and DOX, while MX2 was only half as potent as the parent compound. The three morpholinyl drugs were non-cross-resistant in our DOX-selected MDR variants [5].

In order to further investigate the mechanism(s) of action for these compounds, DNA elution studies were conducted and the alkaline elution profiles of ES-2 cells are shown in Fig. 2. After 2-h drug incubations, protein-associated DNA SSBS appeared to peak at 2 µ*M* for DOX, MMDX and MX2, while MRA continued to show a linear increase in DNA SSBs at 5 µ*M*. With the exception of MRA, DOX and its morpholinyl derivatives appeared to reach a maximum number of SSBs at approximately 200 rad equivalents (Table 2). The neutral elution profiles under deproteinated conditions are shown in Fig. 3. Interestingly, MX2 induced the greatest number of protein-associated DNA DSBs in the ES-2 cell line, followed by DOX. MRA and MMDX induced only a fraction of the DSBs as compared to the parent compound or MX2. MX2 at 25 µ*M* surpassed the 5000 cGy control in its ability to cleave DNA. DOX, on the other hand, reached its maximum DSBs at 10 µ*M*, producing approximately 1500 rad equivalents (Table 3). MRA and MMDX both reached a plateau at 10 µ*M* with approximately 300 rad equivalents.

To verify the results found with neutral elution, we employed the technique of AFIGE or pulse-field gel electrophoresis. The same drug concentrations were used in ES-2 cells for 2-h drug incubations in order to determine the percentage of DNA released under deproteinated conditions. The pulse-field gel profiles substantiated our elution data (Fig. 4), demonstrating that MX2 was clearly the most potent in causing DNA DSBs. DOX-induced DSBs were significant, while strand breaks in ES-2 cells treated with MRA and MMDX were hardly detectable in this assay. In these experiments, however, all compounds appeared to have induced the maximum number of DSBs at 5 µ*M*, with 18% DNA released from the agarose plugs in cells treated with MX2 (Table 4). DOX resulted in approximately 14% DNA released, while both MRA and MMDX released only 1.8 and 1.6%, respectively. Although this assay substantiated our results in terms of the total amount of DNA released post-drug treatment,

Table 1 Cytotoxicity of DOX and the morpholinyl anthracyclines in the ES-2 cell line after 48 h drug exposure. Each IC_{50} value represents the mean of at least four experiments ± SD

Drug	IC_{50} (n*M*)[1]	Potency ratio[a]
DOX	78 ± 7.0	1.0
MRA	29 ± 5.0	2.7
MMDX	2.7 ± 0.3	29
MX2	170 ± 19	0.5

[a] Relative to the IC_{50} for DOX

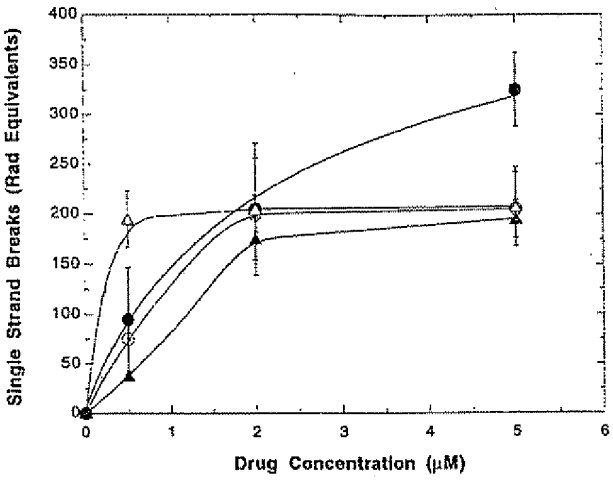


Fig. 2 The alkaline elution (pH 12.1) profiles of ES-2 cells treated with DOX (O), MRA (O), MMDX (Δ) or MX2 (Δ) under deproteinated conditions

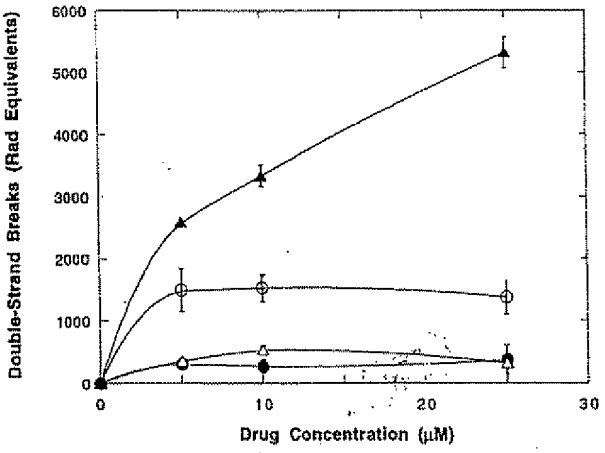


Fig. 3 The neutral elution (pH 9.6) profiles of ES-2 cells treated with DOX (O), MRA (O), MMDX (Δ) or MX2 (Δ) under deproteinated conditions

Table 3 DNA double strand breaks induced in the ES-2 cell line after 2-h exposures to DOX and the morpholinyl anthracyclines. The data are expressed as rad equivalents. Each value represents the mean of at least two experiments ± SD

	Drug			
Concentration (μ*M*)	DOX	MRA	MMDX	MX2
5	1490 ± 350	306 ± 31	355 ± 32	2590 ± 19
10	1520 ± 220	270 ± 110	529 ± 71	3340 ± 180
25	1370 ± 270	370 ± 230	315 ± 76	5320 ± 250

Table 2 DNA single strand breaks induced in the ES-2 cell line after 2-h exposures to DOX and the morpholinyl anthracyclines. The data are expressed as rad equivalents. Each value represents the mean of at least two experiments ± SD

	Drug			
Concentration (μ*M*)	DOX	MRA	MMDX	MX2
0.5	78 ± 1.4	92 ± 52	200 ± 28	39 ± 2.1
2.0	200 ± 18	210 ± 51	210 ± 66	170 ± 21
5.0	220 ± 37	340 ± 37	220 ± 40	200 ± 19

the neutral elution technique provided a better method of quantitating the degree of DNA DSB produced by these compounds because of its ability to measure DNA DSB over a time course (five fractions over 10 h). In this way, one could detect subtle differences after various stages of DNA elution impossible using the AFIGE technique.

Therefore, DOX, MX2, MRA and MMDX all cause protein-associated DNA SSBs to the same degree, but MX2 and DOX are more potent at causing DNA DSBs. These results suggest that MRA and MMDX may cause DNA strand breaks through interaction with Topo I, and that both MX2 and DOX interact with Topo II. K-SDS assays for the quantification of covalently linked Topo I- and II-DNA precipitates were consistent with this hypothesis. MRA and MMDX precipitated significant amounts of DNA linked to protein in whole ES-2 cell preparations relative to CPT, a compound known to interact with Topo I (Fig. 5). Likewise, MX2 and DOX precipitated significant amounts of DNA relative to VP16 used as a positive control.

Further, topoisomerase drug screening assays also suggested that MRA and MMDX interact with Topo I. MRA and MMDX stimulated the formation of cleavable complexes in a dose-dependent fashion, resulting in an increase in the amount of nicked, open circular form of DNA. Also, these drugs inhibited the conversion of supercoiled (form I) *pHOT* DNA to relaxed DNA topoisomers normally seen after Topo I incubation (Fig. 6), while incubation with DOX and MX2 had no effect at the same drug concentrations. Treatment with CPT resulted in an increase in the amount of open circular DNA from relaxed topoisomers at 0.1 m*M*.

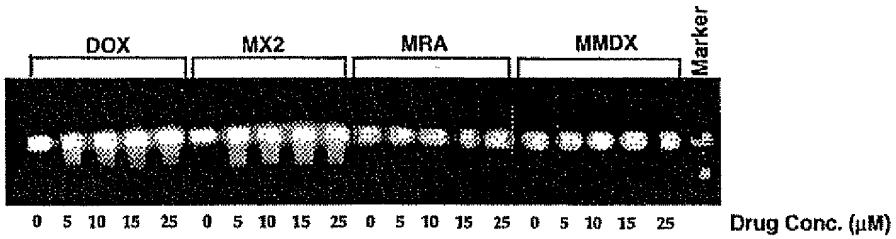


Fig. 4 Pulse-field gel electrophoresis analysis of ES-2 cells treated for 2 h with DOX or the morpholinyl anthracyclines

Table 4 DNA double strand breaks induced in the ES-2 cell line after 2-H exposures to DOX and the morpholinyl anthracyclines analyzed by pulse field gel electrophoresis. The data are expressed as the percentage of labeled DNA released from the loading well into the gel. Each value represents the mean ± SD of at least three experiments

	Drug			
Concentration (μM)	DOX	MRA	MMDX	MX2
5	14.2 ± 0.5	1.8 ± 0.4	1.5 ± 0.2	17.8 ± 1.6
10	11.8 ± 8.4	1.7 ± 0.7	1.4 ± 0.1	14.2 ± 1.9
25	12.0 ± 0.6	1.2 ± 0.5	1.9 ± 0.7	15.3 ± 2.5

In the Topo II assays, DOX and MX2 inhibited the complete conversion of form I DNA to relaxed topoisomers, resulting in an increase in linear DNA stimulated by the formation of cleavable complexes when incubated with the purified human enzyme in the presence of supercoiled *pRYG* DNA (Fig. 7). VM-26, another known Topo II inhibitor was used as a positive control. Treatment with this compound resulted in cleavable complex formation in the form of linear DNA at a concentration of 0.1 μM. At lower concentrations (~ 0.1 μM), treatment with DOX and the morpholinyl anthracyclines resulted in the formation of cleavable complexes, whereas higher concentrations (0.5 to 1.0 μM) prevented the enzyme from binding to DNA resulting in supercoiled form I DNA.

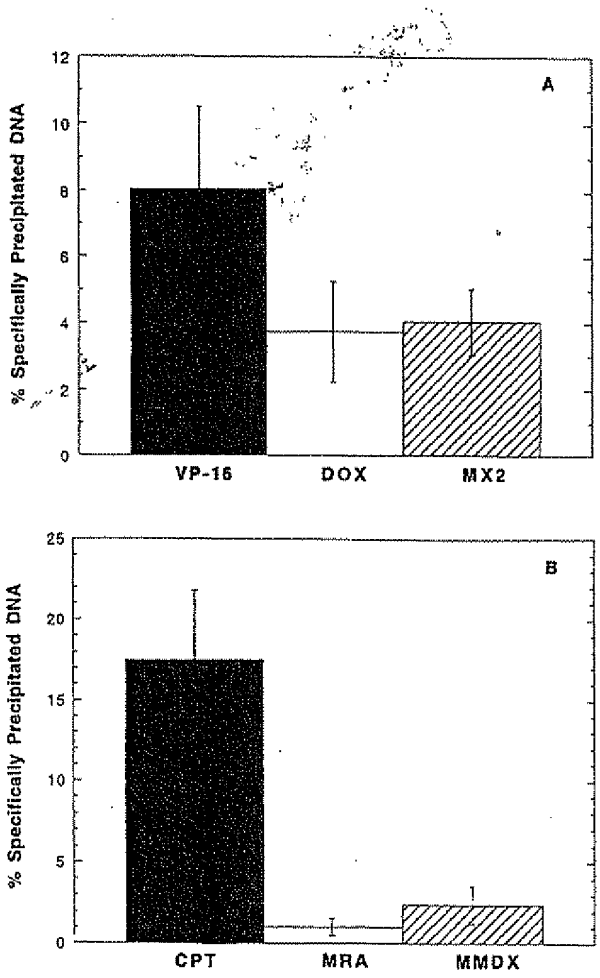


Fig. 5A, B Formation of stabilized cleavable complexes in ES-2 cells exposed to 10 μM of the appropriate drug for 1 h at 37°C as described in the Materials and methods. A VP-16, DOX and MX2; or B CPT, MRA and MMDX. These data are expressed as the mean of four independent experiments calculated as the percentage of DNA specifically precipitated

Discussion

Extensive studies of the effects of DOX on DNA have revealed that it binds to DNA via intercalation [19], subsequently poisoning Topo II [20] and causing protein-associated DNA strand breakage [21]. Several DOX analogues have been synthesized to overcome its cardiotoxic and myelosuppressive effects upon clinical administration. Some analogues, especially those with substitutions at the 3′ amino position of the daunosamine sugar, also show a decreased affinity for the drug transport pump, P-glycoprotein, improving intracellular drug accumulation and in vitro cytotoxicity in MDR models [22].

In the present study, the DNA SSB produced by MMDX and MRA differed significantly from that of the parent compound and MX2. MMDX induced

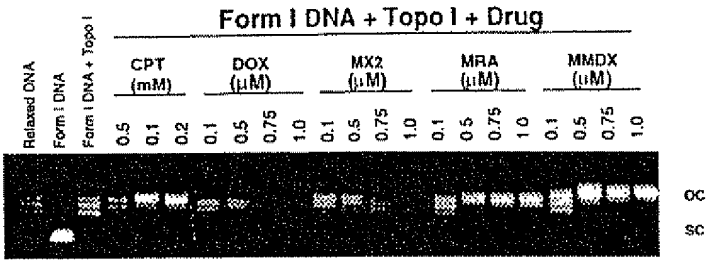


Fig. 6 *pHOT* plasmid (form I DNA) exposed to human topoisomerase I ± DOX or the morpholinyl anthracyclines (0.1, 0.5, 0.75 and 1.0 μ*M*). CPT was used as a positive control for Topo I inhibition (0.05 to 0.2 m*M*)

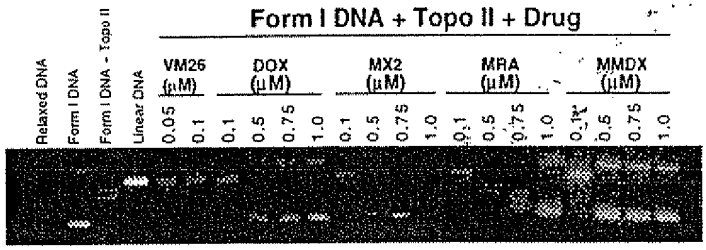


Fig. 7 Supercoiled *pRYG* DNA exposed to human Topo II ± DOX or the morpholinyl anthracyclines (0.1, 0.5, 0.75 and 1.0 μ*M*). VM26 was used as a positive control for Topo II inhibition (0.05 and 0.1 μ*M*)

higher levels of SSB than DOX at lower drug concentrations (Tables 1, 2; Fig. 2). Specifically, MMDX induced 2.5 times higher SSB than DOX at 0.5 μ*M*, which was reflected in MTT assays where MMDX was 29 times more potent than the parent compound. Likewise, MRA induced higher amounts of SSB at the higher concentration of 5.0 μ*M*, and was 2.7 times more potent than DOX after 48 h drug incubation in our cytotoxicity assays.

The MRA and MMDX compounds failed to induce any significant DSB in our neutral elution and pulse-field electrophoresis assays. In contrast, DSBs induced by MX2 and DOX were readily detectable by both techniques. This difference in SSBs and DSBs indicates that MRA and MMDX may work primarily through Topo I since the dominant DNA lesion produced was an SSB. Indeed, our drug inhibition topoisomerase assays provide direct evidence that MRA and MMDX cleavage is associated with Topo I, and that MX2 cleavage is associated with Topo II.

Wasserman et al. have previously reported on the effects of MRA on purified mouse leukemia L1210 DNA Topo I [23]. MRA treatment (1–2 μ*M*) resulted in *fok*I DNA cleavage at position 4955 as well as two novel areas at positions 4975 and 5007 relative to the cleavage pattern usually observed post-CPT exposure. MRA also suppressed cleavage at position 4997 in a manner similar to CPT, with total suppression of Topo I-mediated cleavage at higher concentrations in a dose-dependent manner. In similar assays with purified Topo II, MRA failed to have any effect, while DOX stimulated Topo II cleavage and failed to induce Topo I-associated strand breaks. The cyanomorpholinyl derivative also failed to have any inhibitory effects on this enzyme in the study by Wasserman et al., but stimulated Topo II-mediated cleavage in a manner similar to DOX under the same conditions. The presence of the α-cyano group on the morpholinyl moiety results in a completely different mechanism of action. MRA-CN causes DNA–DNA crosslinks in the ES-2 cell line quite like the bioactivated MRA and MMDX compound upon incubation with human liver microsomes in the presence of NADPH [24]. Further, Capranico et al. found that the morpholinyl group must be at the 3′ position in order to form cleavable complexes since derivatives with the moiety at the 4′ position of DOX failed to stimulate DNA cleavage and trap topoisomerase [25].

There are other fundamental differences between DOX and the morpholino anthracyclines. Data suggest that morpholinyl substitution has a profound effect on ribosomal gene transcription [26]. In contrast to studies with DOX, MRA has been reported to have potent inhibitory effects on ribosomal RNA transcription, while the Topo I inhibitor CPT has been shown to inhibit the synthesis of the 45 S rRNA precursor [27]. These effects have been observed in another antineoplastic agent, actinomycin-D, which induces Topo I-associated DNA strand breaks [23] and has been shown to inhibit ribosomal gene transcription [25]. Thus, there may be a significant correlation between the

effects on Topo I and the inhibitory effects on rRNA transcription.

Finally, since the double-strand lesion is generally considered to be the most lethal, one might expect that MX2 would be the most potent compound in our cytotoxicity testing, since it was the most potent in causing DNA DSBS. Yet, MX2 was only half as potent as DOX in vitro. Although the reasons for this discrepancy are not known, possible explanations include differential repair of MX2 DNA damage and different specificity for DNA sequences or topology. These experiments provide further evidence that minor alterations either on the morpholinyl moiety or on the anthracycline profoundly affect the interaction of these compounds with DNA and topoisomerases.

References

1. Acton EM, Tong GL, Mosher CW, Wolgemuth RL (1984) Intensely potent morpholinyl anthracyclines. J Med Chem 27:638–645
2. Sikic BI, Ehsan MN, Harker WG, Friend NF, Brown BW, Newman RA, Hacker MP, Acton EM (1985) Dissociation of anti-tumor potency from anthracycline cardiotoxicity in a doxorubicin analog. Science 228:1544–1546
3. Streeter DG, Taylor DL, Acton EM, Peters JH (1985) Comparative cytotoxicities of various morpholinyl anthracyclines. Cancer Chemother Pharmacol 14:160–164
4. Streeter, DG, Johl, JS, Gordon GR, Peters JH (1986) Uptake and retention of morpholinyl anthracyclines by adriamycin-sensitive and -resistant P388 cells. Cancer Chemother Pharmacol 16:247–252
5. Lau DHM, Duran GE, Sikic BI (1994) Analysis of intracellular retention of morpholinyl anthracyclines in multidrug resistant cancer cells by interactive laser cytometry. Int J Oncol 5:1273–1277
6. Juliano RL, Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta 455:152–162
7. Gottesman MM, Roninson IB, Pastan I (1989) A molecular basis for multidrug resistance in human cancer cells. In: Kessel D (ed) Resistance to antineoplastic drugs. CRC Press, Boca Raton, pp 19–36
8. Kartner N, Evernden-Porelle D, Bradley G (1985) Detection of P-glycoprotein in multidrug resistant cell lines by monoclonal antibodies. Science 316:820–823
9. Grandi M, Pezzoni G, Ballinari D (1990) Novel anthracycline analogs. Cancer Treat Rev 17: 133–138
10. Watanabe M, Komeshima N, Nakjima S, Tsuruo T (1988) MX2, a morpholino anthracycline, as a new antitumor agent against drug-sensitive and multi-drug sensitive and multidrug-resistant human and murine tumor cells. Cancer Res 48:6653–6657
11. Lau DHM, Duran GE, Lewis AD, Sikic BI (1994) Metabolic conversion of methoxymorpholinyl doxorubicin: from a DNA strand breaker to a DNA cross-linker. Br J Cancer 70: 79–84
12. Lau, DHM, Lewis AD, and Sikic BI (1989) Association of DNA cross-linking with potentiation of the morpholino derivative of doxorubicin by human liver microsomes. J Natl Cancer Inst 81:1034–1038
13. Lewis AD, Lau DHM, Duran GE, Wolf CR, Sikic BI (1992) Role of cytochrome P-450 from the human CYP3A gene family in the potentiation of morpholino doxorubicin by human liver microsomes. Cancer Res: 52: 4379–4384
14. Tracy M, Gordon GR, Nolen HW, Peters JH (1990) Potentiation of the cytotoxicity of morpholinyldoxorubicin (MRA) by human liver microsomes: analytical biochemistry studies. Proc Am Assoc Cancer Res 31:395
15. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
16. Kohn KW (1981) Measurement of strand breaks and cross-links by alkaline elution. In: DNA repair: a laboratory manual of research procedures. Marcel Dekker, New York, pp 379–401
17. Denko P, Giaccia A, Stamato TD (1989) Asymmetric field gel electrophoresis for separation of large DNA pieces. Anal Biochem 178:17–176
18. Rowe TC, Chen GL, Hsiang Y-H (1986) DNA damage by antitumor acridines mediated by mammalian DNA topoisomerase II. Cancer Res 46:2021–2026
19. Quigley GH, Wang AH, Ughetto G, Van der Marel G, Van Boom JH, Rich A (1980) Molecular structure of an anticancer drug-DNA complex: daunomycin plus d (CpGpTpApCpG). Proc Natl Acad Sci USA 77:7204–7208
20. Tewey KM, Rose TC, Yand L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226:466–468
21. Ross WE, Glaubieger DL, Kohn KW (1978) Protein-associated DNA breaks in cells treated with adriamycin or ellipticine. Biochim Biophys Acta 519:23–30
22. Lau DH-M, Duran GE, Sikic BI (1992) Analysis of intracellular uptake of anthracyclines by laser cytometry. Proc Am Assoc Cancer Res 33:468
23. Wasserman K, Markovits J, Jaxel C, Capranico G, Kohn KW, Pommier Y (1990) Effects of morpholinyl doxorubicins, doxorubicin and actinomycin D on mammalian DNA topoisomerase I and II. Mol Pharmacol 38:38–45
24. Jesson MI, Johnston JB, Robotham E, Begleiter A (1989) Characterization of the DNA-DNA cross-linking activity of 3′-(cyano-4-morpholinyl)-deamino-adriamycin. Cancer Res 49:7031–7036
25. Capranico G, Supino R, Binaschi M, Capolongo L, Grandi M, Suarato A, Zunino F (1994) Influence of structural modifications at the 3′ and 4′ positions of doxorubicin on the drug's ability to trap topo II and to overcome multidrug resistance. Mol Pharm 45 (5):908–15.
26. Wassermann K, Newman RA, Davis FM, Mullins TD, Rose KM (1988) Selective inhibition of human ribosomal gene transcription by the morpholinyl anthracyclines cyanomorpholinyl- and morpholinyl-doxorubicin. Cancer Res 48:4101–4106
27. Zhang H, Wang JC, Liu LF (1988) Involvement of DNA topoisomerase I in transcription of human ribosomal RNA genes. Proc Natl Acad Sci USA 85:1060–1064

EXHIBIT 5

Invest New Drugs. 1994; 12(2): 93-7.

Growth-inhibitory properties of novel anthracyclines in human leukemic cell lines expressing either Pgp-MDR or at-MDR.

Mariani M, Capolongo L, Suarato A, Bargiotti A, Mongelli N, Grandi M, Beck WT.

Pharmacia-Farmitalia Carlo Erba, R&D/Experimental Oncology Laboratory, Milano, Italy.

The objective of the experiments reported in this paper was the identification of promising anthracycline analogs on the basis of lack of cross-resistance against tumor cells presenting either P-glycoprotein multidrug resistance (Pgp-MDR) or the altered topoisomerase multidrug resistant (at-MDR) phenotype. Differently modified anthracycline analogs known to be active against MDR cells were assayed in vitro against CEM human leukemic cells, and the sublines CEM/VLB100 and CEM/VM-1 exhibiting respectively the Pgp-MDR and the at-MDR phenotype. Two classes of molecules, in which the -NH2 group in C-3' position is substituted with a morpholino, methoxymorpholino (morpholinyl-anthracycline), or an alkylating moiety, present equivalent efficacy in the drug-sensitive and the two drug-resistant sublines. These results indicate that such molecules may exert their cytotoxic effect through a mode of action different from that of "classical" anthracyclines and is not mediated through topoisomerase II inhibition. Both molecules represent novel concepts in the field of new anthracyclines derivatives.

PMID: 7860237 [PubMed - indexed for MEDLINE]

EXHIBIT 6

UDC 6161.36-006.6-083.277.3-032:611.136 | -036.8-07

V. A. Gorbunova, V. A. Shatikhin, 1990

INTRAHEPATIC ARTERIAL INFUSION CHEMOTHERAPY IN PRIMARY AND METASTATIC HEPATIC CARCINOMA

USSR All-Union Scientific Center for Oncology, Academy of Medical Sciences, Moscow

The objectives of long-term clinical use of arterial chemotherapy in treating liver cancer are: first, tp produce constant cytostatic concentrations and prolong exposure of the tumor in order to enhance the effectiveness of antineoplastic therapy; second, to reduce toxicity of cytostatic therapy by reducing drug concentrations in the blood; third, to use intrahepatic arterial infusion chemotherapy (IAIC) to facilitate the achievement of ultrahigh concentrations of antineoplastic agent in the tumor-affected organ and increase the objective response rate [1, 2, 5].

IAIC is currently widely used to treat patients with primary and metastatic hepatic carcinoma; according to various authors [3-7] its overall effectiveness ranges from 8 to 90%, and averages around 50%.

The effectiveness and toxicity of three IAIC regimes (110 courses) in 60 primary and metastatic liver cancer patients (see table) was studied at the chemotherapy department of the USSR All-Union Scientific Center for Oncology, Academy of Medical Sciences. The patients were from 16 to 90 years old. Prior to beginning IAIC, 42 patients had received adequate regimes of systemic chemotherapy without effect.

Distribution of patients by diagnosis

Clinical diagnosis	Number of patients
Primary hepatic carcinoma	15
Metastatic hepatic carcinoma:	45
breast cancer	18
colon cancer	22
stomach cancer	3
lung carcinoid	1
ovarian cancer	1

All patients had subtotal or total neoplastic involvement of the liver. Laparoscopy, ultrasound, emission computed tomography, angiography and x-ray computed tomography of the liver revealed that the minimum dimensions of neoplastic involvement in the primary cancer patients were 11x11 cm; maximum dimensions were 25x30 cm. A

fundamental consideration is that no patients in this study had tumors less than 5 cm. in size.

To carry out IAIC in the angiography room, percutaneous femoral Seldinger's catheterization was applied, with the distal tip of the catheter placed in the main hepatic artery system. Catheter position was monitored every 48 hours. Uninterrupted arterial delivery of cytostatic agent was accomplished using Soviet-produced type DLV-1 [2] portable drug dosing devices.

The following IAIC regimes were used : for inoperable primary hepatic cell carcinoma and breast carcinoma metastases in the liver -- 72-hour adriamycin infusion at 30 mg/m^2/24 hr (I); in colon carcinoma metastases in the liver -- 96-hour 5-fluorouracil infusion at 1000 mg/m^2/24 (II); as a II line regime when regimes I and II proved ineffective – 100-hour infusion in fibrinolytically active plasma: adriamycin at 50 mg/m^2/24 on day 1, 5-fluorouracil at 1000 mg/m^2/24 on days 2, 3, and 4 and platidiam at 100 mg/m^2/24 on day 5. Platidiam was administered following two hours of intravenous hyperhydration with 1600-2000 ml (III).

Adriamycin and 5-fluorouracil were administered at a rate of about 16-17 ml/hr and platidiam at the rate of 200 ml/hr. The interval between IAIC courses averaged four weeks.

Two vicalin tablets 3 times per day were prescribed during infusion and for 7-10 days thereafter to prevent local gastric toxicity. The catheter was flushed 2-3 times daily with 5000 ED of heparin solution to prevent thromboses. The lower extremities were stabilized throughout the infusion to prevent the catheter from shifting due to its percutaneous femoral placement.

Efficacy and toxicity were assessed according to WHO recommendations using x-ray computed tomography and liver angiography prior to each course of IAIC. Minimal regression (less than 50%) and stabilization were used to more accurately characterize the dynamics of the disease during treatment.

Twelve patients with primary inoperable hepatic carcinoma who received 17 courses of 72-hour adriamycin infusions were assessed. No objective responses were observed in this group. A minimal short-term effect was recorded in two patients (16%), and stabilization and advancement in five patients (42%). In the case of IAIC with adriamycin, two out of four patients with positive alpha-fetoprotein (AFP) values showed a lowering of those values, while AFP indicators remained unchanged in the other two.

The 72-hour adriamycin IAIC regime was evaluated in 16 patients with hepatic metastases from breast cancer. A partial response was obtained in five patients (31%). Partial regression lasted 9.5 months (4-12 months) on average: in two patients it persisted after +6 and +12 months.

Aside from its regional action, administering adriamycin into the hepatic artery system also possesses systemic antineoplastic effects: two patients displayed long-term partial regression of pulmonary and bone metastases of breast cancer.

The results of using 96-hour intra-arterial infusion of 5-fluorouracil were assessed in 20 patients with metastases of colon cancer in the liver. A partial response was observed in two patients (10%), minimal regression in three (15%), stabilization in eight

(40%) and progression in seven (35%). With 5-fluorouracil IAIC, extrahepatic progression in the form of metastases in the lungs and skeletal bones was noted in 25% of the patients, indicating the need to develop new regimes that prudently combine regional and systemic chemotherapy.

The FAP regime was a II line scheme for 11 patients with hepatic metastases of colon carcinoma and for three breast cancer patients with metastases in the liver who had previously received IAIC with adriamycin (I) and 5-fluorouracil (II) without effect; no objective responses were achieved in these patients. Stabilization was observed in 60% of the patients, and progression in 40%.

For eight patients with primary and metastatic liver cancer the FAP regime was a line I scheme; a partial response, which lasted 12 months, occurred in one patient with inoperable hepatic cell cancer, and minimal effects were recorded in one patient with metastases of gastric carcinoma in the liver.

Regarding the toxicity of the three IAIC regimes, the typical features of general cytostatic toxicity appeared, considering the antineoplastic drugs used. For example, grade II-IV leukopenia was observed in 50% of the patients who underwent IAIC with adriamycin (I), grade II-III alopecia in 100%, stomatitis in 20%, and esophagitis in 10%. After infusion of 5-fluorouracil, grade II leukopenia was noted in 5% of the patients and diarrhea in 14%. When the FAP regime was used, leukopenia was observed in 32% of the patients, gastrointestinal toxicity in 100%, and nephrotoxicity in 23%.

Local IAIC toxicity involving the gastrointestinal tract occurred in two (3%) of the 60 patients in the form of iatrogenic gastric ulcers and acute erosive gastritis after the use of intra-arterial adriamycin infusion. Transient hepatic insufficiency (THI), manifested by suppression of the liver's ability to synthesize protein, should be considered yet another manifestation of local toxicity; it was observed in 33% of the patients. THI was observed in 56% of the patients after the use of adriamycin, in 27% when the FAP regime was used, and in 5% of those subjected to 5-fluorouracil infusion. Scarry stricture of the proper and/or common hepatic artery was observed in three patients (4%): in two patients with metastases of breast cancer in the liver after 72-hour adriamycin infusion and in one with metastases of colon cancer in the liver after a single course of 96-hour 5-fluoracil infusion.

Thrombosis of the hepatic artery occurred in two (3%) of the 60 patients and catheter thrombosis in five (7%). The low incidence of thromboses was largely a result of the technical characteristics of the dosing devices used, which made it possible to infuse at fairly high solution feed rates.

One possible downside of IAIC is the femoral percutaneous placement of the catheter, which requires stabilization of the lower extremities throughout the infusion process in order to prevent the catheter from shifting and forcing the patients to remain in a prone position in bed for the whole time. In general the patients tolerated the restriction of movement fairly well, and the forced hypodynamics did not cause patients to decline further IAIC courses. At the same time, two patients (3%) experienced urinary difficulties that required catheterization.

Findings

1. The 72-hour IAIC regime with adriamycin at the dosage of 30 mg/m^2/24 hr had a moderate therapeutic effect in patients with metastases of breast cancer in the liver (partial regression in 31%) and minimal antineoplastic effects in primary inoperable hepatic carcinoma.

2. The 96-hour IAIC regime with 5-fluorouracil at the dosage of 1 mg/m^2/24 hr, used in patients with colon carcinoma metastases in the liver, was characterized by relatively low efficacy (10%) and toxicity, which suggests the possibility of increasing the total dosage and lengthening the time of intra-arterial infusion.

3. In patients with primary and metastatic liver cancer who had previously undergone IAIC with 5-fluorouracil or adriamycin, the use of intra-arterial infusion in an FAP regime as a II line scheme proved unsuccessful.

4. Manifestations of local IAIC toxicity, catheterization complications and the catheters embedded in vessels for long periods were monitored. Catheter placement rules and techniques were followed, catheter position was checked in a timely fashion, catheter care rules were observed, and gastric protectors were used.

LITERATURE

1. *Borisov V. I., Rusakov I. G., Bogdanova N. V.* // Sov. med.—1986--№ 6—pp. 53-59.
2. *Perevodchikova N. I., Gorbunova V. A., Shatikhin V. A.* // Ibid.—1988--№ 9—pp. 28-31.
3. *Shatikhin V. A.* // Ibid.—1987--№ 6—pp. 119-121.
4. *Shatikhin V. A., Platinskiy L. V., Roshchin E. M.* // Ibid.—1989--№ 6—pp. 36-40.
5. *Lokish J. J.* Cancer Chemotherapy by Infusion—Chicago, 1987.
6. *Malta M., Koga S., Shimtzu N.* // Europ. J. Cancer clin. Oncol.—1988—Vol. 24, № 7—pp. 1199-1203.
7. *Nerenstone S. R., IndeD.C., Friedman M. A.* // Cancer Treatm. Rev.—1988—Vol. 15, № 1—pp. 1-25.

Submitted on 24.08.89